

COMPOSITIONS AND METHODS FOR THE THERAPY  
AND DIAGNOSIS OF LUNG CANCER

5 CROSS REFERENCE TO RELATED APPLICATIONS

This application is related to U.S. Patent Application No. 09/738,973, filed December 14, 2000; U.S. Patent Application No. 09/704,512, filed November 1, 2000; U.S. Patent Application No. 09/667,170, filed September 20, 2000; U.S. Provisional Application No. 60/229,763, filed September 1, 2000; U.S. Patent Application No. 09/640,878, filed 10 August 18, 2000; U.S. Patent Application No. 09/588,937, filed June 5, 2000; U.S. Patent Application No. 09/538,037, filed March 29, 2000; U.S. Patent Application No. 09/518,809, filed March 3, 2000; U.S. Patent Application No. 09/476,235 filed December 30, 1999; U.S. Patent Application No. 09/370,838, filed August 9, 1999; and U.S. Patent Application No. 09/285,323, filed April 2, 1999, each a CIP of the previous application and 15 all pending, and incorporated herein by reference.

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to therapy and diagnosis of cancer, such as lung cancer. The invention is more specifically related to polypeptides, comprising at least a portion of a lung tumor protein, and to polynucleotides encoding such 20 polypeptides. Such polypeptides and polynucleotides are useful in pharmaceutical compositions, *e.g.*, vaccines, and other compositions for the diagnosis and treatment of lung cancer.

BACKGROUND OF THE INVENTION

Lung cancer is the primary cause of cancer death among both men and 25 women in the U.S., with an estimated 172,000 new cases being reported in 1994. The five-year survival rate among all lung cancer patients, regardless of the stage of disease at diagnosis, is only 13%. This contrasts with a five-year survival rate of 46% among cases

detected while the disease is still localized. However, only 16% of lung cancers are discovered before the disease has spread.

Early detection is difficult since clinical symptoms are often not seen until the disease has reached an advanced stage. Currently, diagnosis is aided by the use of chest x-rays, analysis of the type of cells contained in sputum and fiberoptic examination of the bronchial passages. Treatment regimens are determined by the type and stage of the cancer, and include surgery, radiation therapy and/or chemotherapy. In spite of considerable research into therapies for the disease, lung cancer remains difficult to treat.

Accordingly, there remains a need in the art for improved vaccines, treatment methods and diagnostic techniques for lung cancer.

### SUMMARY OF THE INVENTION

In one aspect, the present invention provides polynucleotide compositions comprising a sequence selected from the group consisting of:

- (a) sequences provided in SEQ ID NO: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732;
- (b) complements of the sequences provided in SEQ ID NO: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732;
- (c) sequences consisting of at least 20 contiguous residues of a sequence provided in SEQ ID NO: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732;
- (d) sequences that hybridize to a sequence provided in SEQ ID NO: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732, under moderately stringent conditions;
- (e) sequences having at least 75% identity to a sequence of SEQ ID NO: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732;
- (f) sequences having at least 90% identity to a sequence of SEQ ID NO: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732; and

(g) degenerate variants of a sequence provided in SEQ ID NO: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732.

In one preferred embodiment, the polynucleotide compositions of the invention are expressed in at least about 20%, more preferably in at least about 30%, and most preferably in at least about 50% of lung tumors samples tested, at a level that is at least about 2-fold, preferably at least about 5-fold, and most preferably at least about 10-fold higher than that for normal tissues.

The present invention, in another aspect, provides polypeptide compositions comprising an amino acid sequence that is encoded by a polynucleotide sequence described above.

In specific embodiments, the present invention provides polypeptide compositions comprising an amino acid sequence selected from the group consisting of sequences recited in SEQ ID NO: 391, 393, 395, 397, 421, 425-427, 434-439 and 584-587.

In certain preferred embodiments, the polypeptides and/or polynucleotides of the present invention are immunogenic, *i.e.*, they are capable of eliciting an immune response, particularly a humoral and/or cellular immune response, as further described herein.

The present invention further provides fragments, variants and/or derivatives of the disclosed polypeptide and/or polynucleotide sequences, wherein the fragments, variants and/or derivatives preferably have a level of immunogenic activity of at least about 50%, preferably at least about 70% and more preferably at least about 90% of the level of immunogenic activity of a polypeptide sequence set forth in SEQ ID NOs: 391, 393, 395, 397, 421, 425-427, 434-439, 584-587 and or a polypeptide sequence encoded by a polynucleotide sequence set forth in SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433 440-583 and 588-732.

The present invention further provides polynucleotides that encode a polypeptide described above, expression vectors comprising such polynucleotides and host cells transformed or transfected with such expression vectors.

Within other aspects, the present invention provides pharmaceutical compositions comprising a polypeptide or polynucleotide as described above and a physiologically acceptable carrier.

5      Within a related aspect of the present invention, the pharmaceutical compositions, *e.g.*, vaccine compositions, are provided for prophylactic or therapeutic applications. Such compositions generally comprise an immunogenic polypeptide or polynucleotide of the invention and an immunostimulant, such as an adjuvant.

10      The present invention further provides pharmaceutical compositions that comprise: (a) an antibody or antigen-binding fragment thereof that specifically binds to a polypeptide of the present invention, or a fragment thereof; and (b) a physiologically acceptable carrier.

15      Within further aspects, the present invention provides pharmaceutical compositions comprising: (a) an antigen presenting cell that expresses a polypeptide as described above and (b) a pharmaceutically acceptable carrier or excipient. Illustrative antigen presenting cells include dendritic cells, macrophages, monocytes, fibroblasts and B cells.

20      Within related aspects, pharmaceutical compositions are provided that comprise: (a) an antigen presenting cell that expresses a polypeptide as described above and (b) an immunostimulant.

25      The present invention further provides, in other aspects, fusion proteins that comprise at least one polypeptide as described above, as well as polynucleotides encoding such fusion proteins, typically in the form of pharmaceutical compositions, *e.g.*, vaccine compositions, comprising a physiologically acceptable carrier and/or an immunostimulant. The fusions proteins may comprise multiple immunogenic polypeptides or portions/variants thereof, as described herein, and may further comprise one or more polypeptide segments for facilitating the expression, purification and/or immunogenicity of the polypeptide(s).

Within further aspects, the present invention provides methods for stimulating an immune response in a patient, preferably a T cell response in a human patient, comprising administering a pharmaceutical composition described herein. The



patient may be afflicted with lung cancer, in which case the methods provide treatment for the disease, or patient considered at risk for such a disease may be treated prophylactically.

Within further aspects, the present invention provides methods for inhibiting the development of a cancer in a patient, comprising administering to a patient a pharmaceutical composition as recited above. The patient may be afflicted with lung cancer, in which case the methods provide treatment for the disease, or patient considered at risk for such a disease may be treated prophylactically.

The present invention further provides, within other aspects, methods for removing tumor cells from a biological sample, comprising contacting a biological sample with T cells that specifically react with a polypeptide of the present invention, wherein the step of contacting is performed under conditions and for a time sufficient to permit the removal of cells expressing the protein from the sample.

Within related aspects, methods are provided for inhibiting the development of a cancer in a patient, comprising administering to a patient a biological sample treated as described above.

Methods are further provided, within other aspects, for stimulating and/or expanding T cells specific for a polypeptide of the present invention, comprising contacting T cells with one or more of: (i) a polypeptide as described above; (ii) a polynucleotide encoding such a polypeptide; and/or (iii) an antigen presenting cell that expresses such a polypeptide; under conditions and for a time sufficient to permit the stimulation and/or expansion of T cells. Isolated T cell populations comprising T cells prepared as described above are also provided.

Within further aspects, the present invention provides methods for inhibiting the development of a cancer in a patient, comprising administering to a patient an effective amount of a T cell population as described above.

The present invention further provides methods for inhibiting the development of a cancer in a patient, comprising the steps of: (a) incubating CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells isolated from a patient with one or more of: (i) a polypeptide comprising at least an immunogenic portion of polypeptide disclosed herein; (ii) a polynucleotide

encoding such a polypeptide; and (iii) an antigen-presenting cell that expressed such a polypeptide; and (b) administering to the patient an effective amount of the proliferated T cells, and thereby inhibiting the development of a cancer in the patient. Proliferated cells may, but need not, be cloned prior to administration to the patient.

5        Within further aspects, the present invention provides methods for determining the presence or absence of a cancer, preferably a lung cancer, in a patient comprising: (a) contacting a biological sample obtained from a patient with a binding agent that binds to a polypeptide as recited above; (b) detecting in the sample an amount of polypeptide that binds to the binding agent; and (c) comparing the amount of polypeptide  
10        with a predetermined cut-off value, and therefrom determining the presence or absence of a cancer in the patient. Within preferred embodiments, the binding agent is an antibody, more preferably a monoclonal antibody.

The present invention also provides, within other aspects, methods for monitoring the progression of a cancer in a patient. Such methods comprise the steps of:  
15        (a) contacting a biological sample obtained from a patient at a first point in time with a binding agent that binds to a polypeptide as recited above; (b) detecting in the sample an amount of polypeptide that binds to the binding agent; (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d)  
20        comparing the amount of polypeptide detected in step (c) with the amount detected in step (b) and therefrom monitoring the progression of the cancer in the patient.

The present invention further provides, within other aspects, methods for determining the presence or absence of a cancer in a patient, comprising the steps of: (a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a polypeptide of the present invention; (b)  
25        detecting in the sample a level of a polynucleotide, preferably mRNA, that hybridizes to the oligonucleotide; and (c) comparing the level of polynucleotide that hybridizes to the oligonucleotide with a predetermined cut-off value, and therefrom determining the presence or absence of a cancer in the patient. Within certain embodiments, the amount of mRNA is detected via polymerase chain reaction using, for example, at least one oligonucleotide

primer that hybridizes to a polynucleotide encoding a polypeptide as recited above, or a complement of such a polynucleotide. Within other embodiments, the amount of mRNA is detected using a hybridization technique, employing an oligonucleotide probe that hybridizes to a polynucleotide that encodes a polypeptide as recited above, or a complement of such a polynucleotide.

In related aspects, methods are provided for monitoring the progression of a cancer in a patient, comprising the steps of: (a) contacting a biological sample obtained from a patient with an oligonucleotide that hybridizes to a polynucleotide that encodes a polypeptide of the present invention; (b) detecting in the sample an amount of a polynucleotide that hybridizes to the oligonucleotide; (c) repeating steps (a) and (b) using a biological sample obtained from the patient at a subsequent point in time; and (d) comparing the amount of polynucleotide detected in step (c) with the amount detected in step (b) and therefrom monitoring the progression of the cancer in the patient.

Within further aspects, the present invention provides antibodies, such as monoclonal antibodies, that bind to a polypeptide as described above, as well as diagnostic kits comprising such antibodies. Diagnostic kits comprising one or more oligonucleotide probes or primers as described above are also provided.

These and other aspects of the present invention will become apparent upon reference to the following detailed description. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

#### SEQUENCE IDENTIFIERS

SEQ ID NO: 1 is the determined cDNA sequence for L363C1.cons  
 SEQ ID NO: 2 is the determined cDNA sequence for L263C2.cons  
 SEQ ID NO: 3 is the determined cDNA sequence for L263C2c  
 SEQ ID NO: 4 is the determined cDNA sequence for L263C1.cons  
 SEQ ID NO: 5 is the determined cDNA sequence for L263C1b  
 SEQ ID NO: 6 is the determined cDNA sequence for L164C2.cons

SEQ ID NO: 7 is the determined cDNA sequence for L164C1.cons  
SEQ ID NO: 8 is the determined cDNA sequence for L366C1a  
SEQ ID NO: 9 is the determined cDNA sequence for L260C1.cons  
SEQ ID NO: 10 is the determined cDNA sequence for L163C1c  
SEQ ID NO: 11 is the determined cDNA sequence for L163C1b  
5 SEQ ID NO: 12 is the determined cDNA sequence for L255C1.cons  
SEQ ID NO: 13 is the determined cDNA sequence for L255C1b  
SEQ ID NO: 14 is the determined cDNA sequence for L355C1.cons  
SEQ ID NO: 15 is the determined cDNA sequence for L366C1.cons  
10 SEQ ID NO: 16 is the determined cDNA sequence for L163C1a  
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SEQ ID NO: 18 is the determined cDNA sequence for LT86-2  
SEQ ID NO: 19 is the determined cDNA sequence for LT86-3  
SEQ ID NO: 20 is the determined cDNA sequence for LT86-4  
15 SEQ ID NO: 21 is the determined cDNA sequence for LT86-5  
SEQ ID NO: 22 is the determined cDNA sequence for LT86-6  
SEQ ID NO: 23 is the determined cDNA sequence for LT86-7  
SEQ ID NO: 24 is the determined cDNA sequence for LT86-8  
SEQ ID NO: 25 is the determined cDNA sequence for LT86-9  
20 SEQ ID NO: 26 is the determined cDNA sequence for LT86-10  
SEQ ID NO: 27 is the determined cDNA sequence for LT86-11  
SEQ ID NO: 28 is the determined cDNA sequence for LT86-12  
SEQ ID NO: 29 is the determined cDNA sequence for LT86-13  
SEQ ID NO: 30 is the determined cDNA sequence for LT86-14  
25 SEQ ID NO: 31 is the determined cDNA sequence for LT86-15  
SEQ ID NO: 32 is the predicted amino acid sequence for LT86-1  
SEQ ID NO: 33 is the predicted amino acid sequence for LT86-2  
SEQ ID NO: 34 is the predicted amino acid sequence for LT86-3  
SEQ ID NO: 35 is the predicted amino acid sequence for LT86-4

SEQ ID NO: 36 is the predicted amino acid sequence for LT86-5

SEQ ID NO: 37 is the predicted amino acid sequence for LT86-6

SEQ ID NO: 38 is the predicted amino acid sequence for LT86-7

SEQ ID NO: 39 is the predicted amino acid sequence for LT86-8

SEQ ID NO: 40 is the predicted amino acid sequence for LT86-9

SEQ ID NO: 41 is the predicted amino acid sequence for LT86-10

SEQ ID NO: 42 is the predicted amino acid sequence for LT86-11

SEQ ID NO: 43 is the predicted amino acid sequence for LT86-12

SEQ ID NO: 44 is the predicted amino acid sequence for LT86-13

SEQ ID NO: 45 is the predicted amino acid sequence for LT86-14

SEQ ID NO: 46 is the predicted amino acid sequence for LT86-15

SEQ ID NO: 47 is a (dT)<sub>12</sub>AG primer

SEQ ID NO: 48 is a primer

SEQ ID NO: 49 is the determined 5' cDNA sequence for L86S-3

SEQ ID NO: 50 is the determined 5' cDNA sequence for L86S-12

SEQ ID NO: 51 is the determined 5' cDNA sequence for L86S-16

SEQ ID NO: 52 is the determined 5' cDNA sequence for L86S-25

SEQ ID NO: 53 is the determined 5' cDNA sequence for L86S-36

SEQ ID NO: 54 is the determined 5' cDNA sequence for L86S-40

SEQ ID NO: 55 is the determined 5' cDNA sequence for L86S-46

SEQ ID NO: 56 is the predicted amino acid sequence for L86S-3

SEQ ID NO: 57 is the predicted amino acid sequence for L86S-12

SEQ ID NO: 58 is the predicted amino acid sequence for L86S-16

SEQ ID NO: 59 is the predicted amino acid sequence for L86S-25

SEQ ID NO: 60 is the predicted amino acid sequence for L86S-36

SEQ ID NO: 61 is the predicted amino acid sequence for L86S-40

SEQ ID NO: 62 is the predicted amino acid sequence for L86S-46

SEQ ID NO: 63 is the determined 5' cDNA sequence for L86S-30

SEQ ID NO: 64 is the determined 5' cDNA sequence for L86S-41

SEQ ID NO: 65 is the predicted amino acid sequence from the 5' end of

LT86-9

SEQ ID NO: 66 is the determined extended cDNA sequence for LT86-4

SEQ ID NO: 67 is the predicted extended amino acid sequence for LT86-4

SEQ ID NO: 68 is the determined 5' cDNA sequence for LT86-20

SEQ ID NO: 69 is the determined 3' cDNA sequence for LT86-21

SEQ ID NO: 70 is the determined 5' cDNA sequence for LT86-22

SEQ ID NO: 71 is the determined 5' cDNA sequence for LT86-26

SEQ ID NO: 72 is the determined 5' cDNA sequence for LT86-27

SEQ ID NO: 73 is the predicted amino acid sequence for LT86-20

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SEQ ID NO: 75 is the predicted amino acid sequence for LT86-22

SEQ ID NO: 76 is the predicted amino acid sequence for LT86-26

SEQ ID NO: 77 is the predicted amino acid sequence for LT86-27

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SEQ ID NO: 79 is the determined extended cDNA sequence for L86S-36

SEQ ID NO: 80 is the determined extended cDNA sequence for L86S-46

SEQ ID NO: 81 is the predicted extended amino acid sequence for L86S-12

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SEQ ID NO: 86 is the determined 5'cDNA sequence for L86S-14

SEQ ID NO: 87 is the determined 5'cDNA sequence for L86S-29

SEQ ID NO: 88 is the determined 5'cDNA sequence for L86S-34

SEQ ID NO: 89 is the determined 5'cDNA sequence for L86S-39

SEQ ID NO: 90 is the determined 5'cDNA sequence for L86S-47

SEQ ID NO: 91 is the determined 5'cDNA sequence for L86S-49

SEQ ID NO: 92 is the determined 5'cDNA sequence for L86S-51

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SEQ ID NO: 107 is the determined 5' cDNA sequence for SLT-T9  
SEQ ID NO: 108 is the determined 5' cDNA sequence for SLT-T10  
SEQ ID NO: 109 is the determined 5' cDNA sequence for SLT-T11  
SEQ ID NO: 110 is the determined 5' cDNA sequence for SLT-T12  
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SEQ ID NO: 113 is the predicted amino acid sequence for SLT-T3  
SEQ ID NO: 114 is the predicted amino acid sequence for SLT-T10  
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SEQ ID NO: 140 is the determined cDNA sequence for PSLT-011  
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SEQ ID NO: 145 is the determined cDNA sequence for PSLT-74  
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SEQ ID NO: 148 is the determined cDNA sequence for PSLT-037  
SEQ ID NO: 149 is the determined 5' cDNA sequence for SAL-3  
SEQ ID NO: 150 is the determined 5' cDNA sequence for SAL-24



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SEQ ID NO: 178 is the determined 5' cDNA sequence for SAL-88  
SEQ ID NO: 179 is the determined 5' cDNA sequence for SAL-93

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SEQ ID NO: 189 is a first predicted amino acid sequence for SAL-66  
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SEQ ID NO: 191 is the predicted amino acid sequence for SAL-82  
SEQ ID NO: 192 is the predicted amino acid sequence for SAL-99  
SEQ ID NO: 193 is the predicted amino acid sequence for SAL-104  
SEQ ID NO: 194 is the predicted amino acid sequence for SAL-5  
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SEQ ID NO: 213 is the predicted amino acid sequence for SAL-93  
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25 SEQ ID NO: 233 is the determined 3' cDNA sequence for LT4690-55  
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5 SEQ ID NO: 238 is the determined cDNA sequence for 2LT-3  
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SEQ ID NO: 263 is the determined cDNA sequence for 2LT-85  
SEQ ID NO: 264 is the determined cDNA sequence for 2LT-87  
SEQ ID NO: 265 is the determined cDNA sequence for 2LT-89  
SEQ ID NO: 266 is the determined cDNA sequence for 2LT-94

SEQ ID NO: 267 is the determined cDNA sequence for 2LT-95  
SEQ ID NO: 268 is the determined cDNA sequence for 2LT-98  
SEQ ID NO: 269 is the determined cDNA sequence for 2LT-100  
SEQ ID NO: 270 is the determined cDNA sequence for 2LT-103  
5 SEQ ID NO: 271 is the determined cDNA sequence for 2LT-105  
SEQ ID NO: 272 is the determined cDNA sequence for 2LT-107  
SEQ ID NO: 273 is the determined cDNA sequence for 2LT-108  
SEQ ID NO: 274 is the determined cDNA sequence for 2LT-109  
SEQ ID NO: 275 is the determined cDNA sequence for 2LT-118  
10 SEQ ID NO: 276 is the determined cDNA sequence for 2LT-120  
SEQ ID NO: 277 is the determined cDNA sequence for 2LT-121  
SEQ ID NO: 278 is the determined cDNA sequence for 2LT-122  
SEQ ID NO: 279 is the determined cDNA sequence for 2LT-124  
SEQ ID NO: 280 is the determined cDNA sequence for 2LT-126  
15 SEQ ID NO: 281 is the determined cDNA sequence for 2LT-127  
SEQ ID NO: 282 is the determined cDNA sequence for 2LT-128  
SEQ ID NO: 283 is the determined cDNA sequence for 2LT-129  
SEQ ID NO: 284 is the determined cDNA sequence for 2LT-133  
SEQ ID NO: 285 is the determined cDNA sequence for 2LT-137  
20 SEQ ID NO: 286 is the determined cDNA sequence for LT4690-71  
SEQ ID NO: 287 is the determined cDNA sequence for LT4690-82  
SEQ ID NO: 288 is the determined full-length cDNA sequence for SSLT-74  
SEQ ID NO: 289 is the determined cDNA sequence for SSLT-78  
SEQ ID NO: 290 is the determined cDNA sequence for SCC1-8.  
25 SEQ ID NO: 291 is the determined cDNA sequence for SCC1-12.  
SEQ ID NO: 292 is the determined cDNA sequence for SCC1-336  
SEQ ID NO: 293 is the determined cDNA sequence for SCC1-344  
SEQ ID NO: 294 is the determined cDNA sequence for SCC1-345  
SEQ ID NO: 295 is the determined cDNA sequence for SCC1-346

5 SEQ ID NO: 296 is the determined cDNA sequence for SCC1-348  
SEQ ID NO: 297 is the determined cDNA sequence for SCC1-350  
SEQ ID NO: 298 is the determined cDNA sequence for SCC1-352  
SEQ ID NO: 299 is the determined cDNA sequence for SCC1-354  
SEQ ID NO: 300 is the determined cDNA sequence for SCC1-355  
SEQ ID NO: 301 is the determined cDNA sequence for SCC1-356  
SEQ ID NO: 302 is the determined cDNA sequence for SCC1-357  
SEQ ID NO: 303 is the determined cDNA sequence for SCC1-501  
SEQ ID NO: 304 is the determined cDNA sequence for SCC1-503  
10 SEQ ID NO: 305 is the determined cDNA sequence for SCC1-513  
SEQ ID NO: 306 is the determined cDNA sequence for SCC1-516  
SEQ ID NO: 307 is the determined cDNA sequence for SCC1-518  
SEQ ID NO: 308 is the determined cDNA sequence for SCC1-519  
SEQ ID NO: 309 is the determined cDNA sequence for SCC1-522  
15 SEQ ID NO: 310 is the determined cDNA sequence for SCC1-523  
SEQ ID NO: 311 is the determined cDNA sequence for SCC1-525  
SEQ ID NO: 312 is the determined cDNA sequence for SCC1-527  
SEQ ID NO: 313 is the determined cDNA sequence for SCC1-529  
SEQ ID NO: 314 is the determined cDNA sequence for SCC1-530  
20 SEQ ID NO: 315 is the determined cDNA sequence for SCC1-531  
SEQ ID NO: 316 is the determined cDNA sequence for SCC1-532  
SEQ ID NO: 317 is the determined cDNA sequence for SCC1-533  
SEQ ID NO: 318 is the determined cDNA sequence for SCC1-536  
SEQ ID NO: 319 is the determined cDNA sequence for SCC1-538  
25 SEQ ID NO: 320 is the determined cDNA sequence for SCC1-539  
SEQ ID NO: 321 is the determined cDNA sequence for SCC1-541  
SEQ ID NO: 322 is the determined cDNA sequence for SCC1-542  
SEQ ID NO: 323 is the determined cDNA sequence for SCC1-546  
SEQ ID NO: 324 is the determined cDNA sequence for SCC1-549

SEQ ID NO: 325 is the determined cDNA sequence for SCC1-551  
SEQ ID NO: 326 is the determined cDNA sequence for SCC1-552  
SEQ ID NO: 327 is the determined cDNA sequence for SCC1-554  
SEQ ID NO: 328 is the determined cDNA sequence for SCC1-558  
5 SEQ ID NO: 329 is the determined cDNA sequence for SCC1-559  
SEQ ID NO: 330 is the determined cDNA sequence for SCC1-561  
SEQ ID NO: 331 is the determined cDNA sequence for SCC1-562  
SEQ ID NO: 332 is the determined cDNA sequence for SCC1-564  
SEQ ID NO: 333 is the determined cDNA sequence for SCC1-565  
10 SEQ ID NO: 334 is the determined cDNA sequence for SCC1-566  
SEQ ID NO: 335 is the determined cDNA sequence for SCC1-567  
SEQ ID NO: 336 is the determined cDNA sequence for SCC1-568  
SEQ ID NO: 337 is the determined cDNA sequence for SCC1-570  
SEQ ID NO: 338 is the determined cDNA sequence for SCC1-572  
15 SEQ ID NO: 339 is the determined cDNA sequence for SCC1-575  
SEQ ID NO: 340 is the determined cDNA sequence for SCC1-576  
SEQ ID NO: 341 is the determined cDNA sequence for SCC1-577  
SEQ ID NO: 342 is the determined cDNA sequence for SCC1-578  
SEQ ID NO: 343 is the determined cDNA sequence for SCC1-582  
20 SEQ ID NO: 344 is the determined cDNA sequence for SCC1-583  
SEQ ID NO: 345 is the determined cDNA sequence for SCC1-586  
SEQ ID NO: 346 is the determined cDNA sequence for SCC1-588  
SEQ ID NO: 347 is the determined cDNA sequence for SCC1-590  
SEQ ID NO: 348 is the determined cDNA sequence for SCC1-591  
25 SEQ ID NO: 349 is the determined cDNA sequence for SCC1-592  
SEQ ID NO: 350 is the determined cDNA sequence for SCC1-593  
SEQ ID NO: 351 is the determined cDNA sequence for SCC1-594  
SEQ ID NO: 352 is the determined cDNA sequence for SCC1-595  
SEQ ID NO: 353 is the determined cDNA sequence for SCC1-596

SEQ ID NO: 354 is the determined cDNA sequence for SCC1-598  
SEQ ID NO: 355 is the determined cDNA sequence for SCC1-599  
SEQ ID NO: 356 is the determined cDNA sequence for SCC1-602  
SEQ ID NO: 357 is the determined cDNA sequence for SCC1-604  
5 SEQ ID NO: 358 is the determined cDNA sequence for SCC1-605  
SEQ ID NO: 359 is the determined cDNA sequence for SCC1-606  
SEQ ID NO: 360 is the determined cDNA sequence for SCC1-607  
SEQ ID NO: 361 is the determined cDNA sequence for SCC1-608  
SEQ ID NO: 362 is the determined cDNA sequence for SCC1-610  
10 SEQ ID NO: 363 is the determined cDNA sequence for clone DMS79T1  
SEQ ID NO: 364 is the determined cDNA sequence for clone DMS79T2  
SEQ ID NO: 365 is the determined cDNA sequence for clone DMS79T3  
SEQ ID NO: 366 is the determined cDNA sequence for clone DMS79T5  
SEQ ID NO: 367 is the determined cDNA sequence for clone DMS79T6  
15 SEQ ID NO: 368 is the determined cDNA sequence for clone DMS79T7  
SEQ ID NO: 369 is the determined cDNA sequence for clone DMS79T9  
SEQ ID NO: 370 is the determined cDNA sequence for clone DMS79T10  
SEQ ID NO: 371 is the determined cDNA sequence for clone DMS79T11  
SEQ ID NO: 372 is the determined cDNA sequence for clone 128T1  
20 SEQ ID NO: 373 is the determined cDNA sequence for clone 128T2  
SEQ ID NO: 374 is the determined cDNA sequence for clone 128T3  
SEQ ID NO: 375 is the determined cDNA sequence for clone 128T4  
SEQ ID NO: 376 is the determined cDNA sequence for clone 128T5  
SEQ ID NO: 377 is the determined cDNA sequence for clone 128T7  
25 SEQ ID NO: 378 is the determined cDNA sequence for clone 128T9  
SEQ ID NO: 379 is the determined cDNA sequence for clone 128T10  
SEQ ID NO: 380 is the determined cDNA sequence for clone 128T11  
SEQ ID NO: 381 is the determined cDNA sequence for clone 128T12  
SEQ ID NO: 382 is the determined cDNA sequence for clone NC1H69T3



SEQ ID NO: 383 is the determined cDNA sequence for clone NCIH69T5  
 SEQ ID NO: 384 is the determined cDNA sequence for clone NCIH69T6  
 SEQ ID NO: 385 is the determined cDNA sequence for clone NCIH69T7  
 SEQ ID NO: 386 is the determined cDNA sequence for clone NCIH69T9  
 SEQ ID NO: 387 is the determined cDNA sequence for clone NCIH69T10  
 SEQ ID NO: 388 is the determined cDNA sequence for clone NCIH69T11  
 SEQ ID NO: 389 is the determined cDNA sequence for clone NCIH69T12  
 SEQ ID NO: 390 is the full-length cDNA sequence for 128T1  
 SEQ ID NO: 391 is the amino acid sequence for 128T1  
 SEQ ID NO: 392 is the full-length cDNA sequence for 2LT-128  
 SEQ ID NO: 393 is the amino acid sequence for 2LT-128  
 SEQ ID NO: 394 is an extended cDNA sequence for clone SCC1-542  
 SEQ ID NO: 395 is the amino acid sequence corresponding to SEQ ID  
 NO:394  
 SEQ ID NO: 396 is an extended cDNA sequence for clone SCC1-593  
 SEQ ID NO: 397 is the amino acid sequence corresponding to SEQ ID  
 NO:396  
 SEQ ID NO:398 is the determined cDNA sequence for 55508.1  
 SEQ ID NO:399 is the determined cDNA sequence for 55509.1  
 SEQ ID NO:400 is the determined cDNA sequence for 54243.1  
 SEQ ID NO:401 is the determined cDNA sequence for 54251.1  
 SEQ ID NO:402 is the determined cDNA sequence for 54252.1  
 SEQ ID NO:403 is the determined cDNA sequence for 54253.1  
 SEQ ID NO:404 is the determined cDNA sequence for 55518.1  
 SEQ ID NO:405 is the determined cDNA sequence for 54258.1  
 SEQ ID NO:406 is the determined cDNA sequence for 54575.1  
 SEQ ID NO:407 is the determined cDNA sequence for 54577.1  
 SEQ ID NO:408 is the determined cDNA sequence for 54584.1  
 SEQ ID NO:409 is the determined cDNA sequence for 55521.1

- 5 SEQ ID NO:410 is the determined cDNA sequence for 54589.1  
 SEQ ID NO:411 is the determined cDNA sequence for 54592.1  
 SEQ ID NO:412 is the determined cDNA sequence for 55134.1  
 SEQ ID NO:413 is the determined cDNA sequence for 55137.1  
 SEQ ID NO:414 is the determined cDNA sequence for 55140.1  
 SEQ ID NO:415 is the determined cDNA sequence for 55531.1  
 SEQ ID NO:416 is the determined cDNA sequence for 55532.1  
 SEQ ID NO:417 is the determined cDNA sequence for 54621.1  
 SEQ ID NO:418 is the determined cDNA sequence for 55548.1  
 10 SEQ ID NO:419 is the determined cDNA sequence for 54623.1  
 SEQ ID NO:420 is the determined cDNA sequence for L39  
 SEQ ID NO:421 is the predicted amino acid sequence for L39  
 SEQ ID NO:422 is the determined cDNA sequence for SCC2-29  
 SEQ ID NO:423 is the determined cDNA sequence for SCC2-36  
 15 SEQ ID NO:424 is the determined cDNA sequence for SCC2-60  
 SEQ ID NO:425 is the predicted amino acid sequence for SCC2-29  
 SEQ ID NO:426 is the predicted amino acid sequence for SCC2-36  
 SEQ ID NO:427 is the predicted amino acid sequence for SCC2-60  
 SEQ ID NO:428 is an extended cDNA sequence for the clone 20129, also  
 20 referred to as 2LT-3, set forth in SEQ ID NO: 238  
 SEQ ID NO:429 is an extended cDNA sequence for the clone 20347, also  
 referred to as 2LT-26, set forth in SEQ ID NO: 242  
 SEQ ID NO:430 is an extended cDNA sequence for the clone 21282, also  
 referred to as 2LT-57, set forth in SEQ ID NO: 249  
 25 SEQ ID NO:431 is an extended cDNA sequence for the clone 21283, also  
 referred to as 2LT-58, set forth in SEQ ID NO: 250  
 SEQ ID NO:432 is an extended cDNA sequence for the clone 21484, also  
 referred to as 2LT-98, set forth in SEQ ID NO: 268

SEQ ID NO:433 is an extended cDNA sequence for the clone 21871, also referred to as 2LT-124, set forth in SEQ ID NO: 279

SEQ ID NO:434 is an amino acid sequence encoded by SEQ ID NO: 428

SEQ ID NO:435 is an amino acid sequence encoded by SEQ ID NO: 429

SEQ ID NO:436 is an amino acid sequence encoded by SEQ ID NO: 430

SEQ ID NO:437 is an amino acid sequence encoded by SEQ ID NO: 431

SEQ ID NO:438 is an amino acid sequence encoded by SEQ ID NO: 432

SEQ ID NO:439 is an amino acid sequence encoded by SEQ ID NO: 433

SEQ ID NO:440 is the determined cDNA sequence for clone 19A4

SEQ ID NO: 441 is the determined full-length cDNA sequence for clone

14F10.

SEQ ID NO: 442 is the determined 5' cDNA sequence for clone 20E10.

SEQ ID NO: 443 is a first determined cDNA sequence for clone 55153.

SEQ ID NO: 444 is a second determined cDNA sequence for clone 55153.

SEQ ID NO: 445 is a first determined cDNA sequence for clone 55154.

SEQ ID NO: 446 is a second determined cDNA sequence for clone 55154.

SEQ ID NO: 447 is the determined cDNA sequence for clone 55155.

SEQ ID NO: 448 is a first determined cDNA sequence for clone 55156.

SEQ ID NO: 449 is a second determined cDNA sequence for clone 55156.

SEQ ID NO: 450 is a first determined cDNA sequence for clone 55157.

SEQ ID NO: 451 is a second determined cDNA sequence for clone 55157.

SEQ ID NO: 452 is the determined cDNA sequence for clone 55158.

SEQ ID NO: 453 is the determined cDNA sequence for clone 55159.

SEQ ID NO: 454 is a first determined cDNA sequence for clone 55161.

SEQ ID NO: 455 is a second determined cDNA sequence for clone 55161.

SEQ ID NO: 456 is a first determined cDNA sequence for clone 55162.

SEQ ID NO: 457 is a second determined cDNA sequence for clone 55162.

SEQ ID NO: 458 is a first determined cDNA sequence for clone 55163.

SEQ ID NO: 459 is a second determined cDNA sequence for clone 55163.

SEQ ID NO: 460 is a first determined cDNA sequence for clone 55164.  
SEQ ID NO: 461 is a second determined cDNA sequence for clone 55164.  
SEQ ID NO: 462 is a first determined cDNA sequence for clone 55165.  
SEQ ID NO: 463 is a second determined cDNA sequence for clone 55165.  
5 SEQ ID NO: 464 is a first determined cDNA sequence for clone 55166.  
SEQ ID NO: 465 is a second determined cDNA sequence for clone 55166.  
SEQ ID NO: 466 is a first determined cDNA sequence for clone 55167.  
SEQ ID NO: 467 is a second determined cDNA sequence for clone 55167.  
SEQ ID NO: 468 is a first determined cDNA sequence for clone 55168.  
10 SEQ ID NO: 469 is a second determined cDNA sequence for clone 55168.  
SEQ ID NO: 470 is a first determined cDNA sequence for clone 55169.  
SEQ ID NO: 471 is a second determined cDNA sequence for clone 55169.  
SEQ ID NO: 472 is a first determined cDNA sequence for clone 55170.  
SEQ ID NO: 473 is a second determined cDNA sequence for clone 55170.  
15 SEQ ID NO: 474 is the determined cDNA sequence for clone 55171.  
SEQ ID NO: 475 is the determined cDNA sequence for clone 55172.  
SEQ ID NO: 476 is the determined cDNA sequence for clone 55173.  
SEQ ID NO: 477 is a first determined cDNA sequence for clone 55174.  
SEQ ID NO: 478 is a second determined cDNA sequence for clone 55174.  
20 SEQ ID NO: 479 is the determined cDNA sequence for clone 55175.  
SEQ ID NO: 480 is the determined cDNA sequence for clone 55176.  
SEQ ID NO: 481 is the determined cDNA sequence for contig 525.  
SEQ ID NO: 482 is the determined cDNA sequence for contig 526.  
SEQ ID NO: 483 is the determined cDNA sequence for contig 527.  
25 SEQ ID NO: 484 is the determined cDNA sequence for contig 528.  
SEQ ID NO: 485 is the determined cDNA sequence for contig 529.  
SEQ ID NO: 486 is the determined cDNA sequence for contig 530.  
SEQ ID NO: 487 is the determined cDNA sequence for contig 531.  
SEQ ID NO: 488 is the determined cDNA sequence for contig 532.

SEQ ID NO: 489 is the determined cDNA sequence for contig 533.  
SEQ ID NO: 490 is the determined cDNA sequence for contig 534.  
SEQ ID NO: 491 is the determined cDNA sequence for contig 535.  
SEQ ID NO: 492 is the determined cDNA sequence for contig 536.  
5 SEQ ID NO: 493 is the determined cDNA sequence for contig 537.  
SEQ ID NO: 494 is the determined cDNA sequence for contig 538.  
SEQ ID NO: 495 is the determined cDNA sequence for contig 539.  
SEQ ID NO: 496 is the determined cDNA sequence for contig 540.  
SEQ ID NO: 497 is the determined cDNA sequence for contig 541.  
10 SEQ ID NO: 498 is the determined cDNA sequence for contig 542.  
SEQ ID NO: 499 is the determined cDNA sequence for contig 543.  
SEQ ID NO: 500 is the determined cDNA sequence for contig 544.  
SEQ ID NO: 501 is the determined cDNA sequence for contig 545.  
SEQ ID NO: 502 is the determined cDNA sequence for contig 546.  
15 SEQ ID NO: 503 is the determined cDNA sequence for contig 547.  
SEQ ID NO: 504 is the determined cDNA sequence for contig 548.  
SEQ ID NO: 505 is the determined cDNA sequence for contig 549.  
SEQ ID NO: 506 is the determined cDNA sequence for contig 550.  
SEQ ID NO: 507 is the determined cDNA sequence for contig 551.  
20 SEQ ID NO: 508 is the determined cDNA sequence for contig 552.  
SEQ ID NO: 509 is the determined cDNA sequence for contig 553.  
SEQ ID NO: 510 is the determined cDNA sequence for contig 554.  
SEQ ID NO: 511 is the determined cDNA sequence for contig 555.  
SEQ ID NO: 512 is the determined cDNA sequence for clone 57207.  
25 SEQ ID NO: 513 is the determined cDNA sequence for clone 57209.  
SEQ ID NO: 514 is the determined cDNA sequence for clone 57210.  
SEQ ID NO: 515 is the determined cDNA sequence for clone 57211.  
SEQ ID NO: 516 is the determined cDNA sequence for clone 57212.  
SEQ ID NO: 517 is the determined cDNA sequence for clone 57213.

SEQ ID NO: 518 is the determined cDNA sequence for clone 57215.  
SEQ ID NO: 519 is the determined cDNA sequence for clone 57219.  
SEQ ID NO: 520 is the determined cDNA sequence for clone 57221.  
SEQ ID NO: 521 is the determined cDNA sequence for clone 57222.  
SEQ ID NO: 522 is the determined cDNA sequence for clone 57223.  
SEQ ID NO: 523 is the determined cDNA sequence for clone 57225.  
SEQ ID NO: 524 is the determined cDNA sequence for clone 57227.  
SEQ ID NO: 525 is the determined cDNA sequence for clone 57228.  
SEQ ID NO: 526 is the determined cDNA sequence for clone 57229.  
SEQ ID NO: 527 is the determined cDNA sequence for clone 57230.  
SEQ ID NO: 528 is the determined cDNA sequence for clone 57231.  
SEQ ID NO: 529 is the determined cDNA sequence for clone 57232.  
SEQ ID NO: 530 is the determined cDNA sequence for clone 57233.  
SEQ ID NO: 531 is the determined cDNA sequence for clone 57234.  
SEQ ID NO: 532 is the determined cDNA sequence for clone 57235.  
SEQ ID NO: 533 is the determined cDNA sequence for clone 57236.  
SEQ ID NO: 534 is the determined cDNA sequence for clone 57237.  
SEQ ID NO: 535 is the determined cDNA sequence for clone 57238.  
SEQ ID NO: 536 is the determined cDNA sequence for clone 57239.  
SEQ ID NO: 537 is the determined cDNA sequence for clone 57240.  
SEQ ID NO: 538 is the determined cDNA sequence for clone 57242.  
SEQ ID NO: 539 is the determined cDNA sequence for clone 57243.  
SEQ ID NO: 540 is the determined cDNA sequence for clone 57245.  
SEQ ID NO: 541 is the determined cDNA sequence for clone 57248.  
SEQ ID NO: 542 is the determined cDNA sequence for clone 57249.  
SEQ ID NO: 543 is the determined cDNA sequence for clone 57250.  
SEQ ID NO: 544 is the determined cDNA sequence for clone 57251.  
SEQ ID NO: 545 is the determined cDNA sequence for clone 57253.  
SEQ ID NO: 546 is the determined cDNA sequence for clone 57254.

SEQ ID NO: 547 is the determined cDNA sequence for clone 57255.  
SEQ ID NO: 548 is the determined cDNA sequence for clone 57257.  
SEQ ID NO: 549 is the determined cDNA sequence for clone 57258.  
SEQ ID NO: 550 is the determined cDNA sequence for clone 57259.  
SEQ ID NO: 551 is the determined cDNA sequence for clone 57261.  
SEQ ID NO: 552 is the determined cDNA sequence for clone 57262.  
SEQ ID NO: 553 is the determined cDNA sequence for clone 57263.  
SEQ ID NO: 554 is the determined cDNA sequence for clone 57264.  
SEQ ID NO: 555 is the determined cDNA sequence for clone 57265.  
SEQ ID NO: 556 is the determined cDNA sequence for clone 57266.  
SEQ ID NO: 557 is the determined cDNA sequence for clone 57267.  
SEQ ID NO: 558 is the determined cDNA sequence for clone 57268.  
SEQ ID NO: 559 is the determined cDNA sequence for clone 57269.  
SEQ ID NO: 560 is the determined cDNA sequence for clone 57270.  
SEQ ID NO: 561 is the determined cDNA sequence for clone 57271.  
SEQ ID NO: 562 is the determined cDNA sequence for clone 57272.  
SEQ ID NO: 563 is the determined cDNA sequence for clone 57274.  
SEQ ID NO: 564 is the determined cDNA sequence for clone 57275.  
SEQ ID NO: 565 is the determined cDNA sequence for clone 57277.  
SEQ ID NO: 566 is the determined cDNA sequence for clone 57280.  
SEQ ID NO: 567 is the determined cDNA sequence for clone 57281.  
SEQ ID NO: 568 is the determined cDNA sequence for clone 57282.  
SEQ ID NO: 569 is the determined cDNA sequence for clone 57283.  
SEQ ID NO: 570 is the determined cDNA sequence for clone 57285.  
SEQ ID NO: 571 is the determined cDNA sequence for clone 57287.  
SEQ ID NO: 572 is the determined cDNA sequence for clone 57288.  
SEQ ID NO: 573 is the determined cDNA sequence for clone 57289.  
SEQ ID NO: 574 is the determined cDNA sequence for clone 57290.  
SEQ ID NO: 575 is the determined cDNA sequence for clone 57292.

SEQ ID NO: 576 is the determined cDNA sequence for clone 57295.

SEQ ID NO: 577 is the determined cDNA sequence for clone 57296.

SEQ ID NO: 578 is the determined cDNA sequence for clone 57297.

SEQ ID NO: 579 is the determined cDNA sequence for clone 57299.

SEQ ID NO: 580 is the determined cDNA sequence for clone 57301.

SEQ ID NO: 581 is the determined cDNA sequence for clone 57302.

SEQ ID NO: 582 is the determined cDNA sequence for the beta chain of a

lung tumor specific T cell receptor.

SEQ ID NO: 583 is the determined cDNA sequence for the alpha chain of a

10 lung tumor specific T cell receptor.

SEQ ID NO: 584 is the amino acid sequence encoded by SEQ ID NO: 583.

SEQ ID NO: 585 is the amino acid sequence encoded by SEQ ID NO: 582.

SEQ ID NO: 586 is the amino acid sequence encoded by the 5' terminus of

14F10.

15

SEQ ID NO: 587 is the amino acid sequence of a T cell epitope contained

within SEQ ID NO: 586.

SEQ ID NO:588 is the determined cDNA sequence for 54533

SEQ ID NO:589 is the determined cDNA sequence for 54534

SEQ ID NO:590 is the determined cDNA sequence for 54536

SEQ ID NO:591 is the determined cDNA sequence for 54538

SEQ ID NO:592 is the determined cDNA sequence for 54540

SEQ ID NO:593 is the determined cDNA sequence for 55084

SEQ ID NO:594 is the determined cDNA sequence for 55086

SEQ ID NO:595 is the determined cDNA sequence for 54555

SEQ ID NO:596 is the determined cDNA sequence for 54557

SEQ ID NO:597 is the determined cDNA sequence for 54564

SEQ ID NO:598 is the determined cDNA sequence for 55098

SEQ ID NO:599 is the determined cDNA sequence for 55473

SEQ ID NO:600 is the determined cDNA sequence for 55104



SEQ ID NO:601 is the determined cDNA sequence for 55105  
SEQ ID NO:602 is the determined cDNA sequence for 55107  
SEQ ID NO:603 is the determined cDNA sequence for 55108  
SEQ ID NO:604 is the determined cDNA sequence for 55114  
5 SEQ ID NO:605 is the determined cDNA sequence for 55477  
SEQ ID NO:606 is the determined cDNA sequence for 55482  
SEQ ID NO:607 is the determined cDNA sequence for 55483  
SEQ ID NO:608 is the determined cDNA sequence for 55485  
SEQ ID NO:609 is the determined cDNA sequence for 55487  
10 SEQ ID NO:610 is the determined cDNA sequence for 55488  
SEQ ID NO:611 is the determined cDNA sequence for 55087  
SEQ ID NO:612 is the determined cDNA sequence for 55089  
SEQ ID NO:613 is the determined cDNA sequence for 55092  
SEQ ID NO:614 is the determined cDNA sequence for 55093  
15 SEQ ID NO:615 is the determined cDNA sequence for 56926  
SEQ ID NO:616 is the determined cDNA sequence for 56930  
SEQ ID NO:617 is the determined cDNA sequence for 56944  
SEQ ID NO:618 is the determined cDNA sequence for 56945  
SEQ ID NO:619 is the determined cDNA sequence for 55490  
20 SEQ ID NO:620 is the determined cDNA sequence for 55495  
SEQ ID NO:621 is the determined cDNA sequence for 55504  
SEQ ID NO:622 is the determined cDNA sequence for 55506  
SEQ ID NO:623 is the determined cDNA sequence for 56480  
SEQ ID NO:624 is the determined cDNA sequence for 56482  
25 SEQ ID NO:625 is the determined cDNA sequence for 56484  
SEQ ID NO:626 is the determined cDNA sequence for 56487  
SEQ ID NO:627 is the determined cDNA sequence for 56488  
SEQ ID NO:628 is the determined cDNA sequence for 56490  
SEQ ID NO:629 is the determined cDNA sequence for 56493

SEQ ID NO:630 is the determined cDNA sequence for 56494

SEQ ID NO:631 is the determined cDNA sequence for 56495

SEQ ID NO:632 is the determined cDNA sequence for 56499

SEQ ID NO:633 is the determined cDNA sequence for 56517

SEQ ID NO:634 is the determined cDNA sequence for 56952

SEQ ID NO:635 is the determined cDNA sequence for 56953

SEQ ID NO:636 is the determined cDNA sequence for 56959

SEQ ID NO:637 is the determined cDNA sequence for 57139

SEQ ID NO:638 is the determined cDNA sequence for 57078

SEQ ID NO:639 is the determined cDNA sequence for 57092

SEQ ID NO:640 is the determined cDNA sequence for 57099

SEQ ID NO:641 is the determined cDNA sequence for 57100

SEQ ID NO:642 is the determined cDNA sequence for 57105

SEQ ID NO:643 is the determined cDNA sequence for 57111

SEQ ID NO:644 is the determined cDNA sequence for 57117

SEQ ID NO:645 is the determined cDNA sequence for 57121

SEQ ID NO:646 is the determined cDNA sequence for 57124

SEQ ID NO:647 is the determined cDNA sequence for 57125

SEQ ID NO:648-686 are the determined cDNA sequences for the clones

described in Tables 9-10.

SEQ ID NO:687-727 are the determined cDNA sequences for the clones described in Tables 11-13.

SEQ ID NO:728 is the determined full-length cDNA sequence for clone DMS39 (partial sequence given in SEQ ID NO:695).

SEQ ID NO:729 is the determined full-length cDNA sequence for clone DMS126 (partial sequence given in SEQ ID NO:708).

SEQ ID NO:730 is the determined full-length cDNA sequence for clone DMS218 (partial sequence given in SEQ ID NO:720).

SEQ ID NO:731 is the determined full-length cDNA sequence for clone DMS253 (partial sequence given in SEQ ID NO:723).

SEQ ID NO:732 is the determined full-length cDNA sequence for clone LSCC-86 (partial sequence given in SEQ ID NO:665).

5 SEQ ID NO:733 is a first amino acid sequence encoded by SEQ ID NO:732 and designated LSCC-86protein1.

SEQ ID NO:734 is a second amino acid sequence encoded by SEQ ID NO:732 and designated LSCC-86protein2.

10 SEQ ID NO:735 is a third amino acid sequence encoded by SEQ ID NO:732 and designated LSCC-86protein3.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed generally to compositions and their use in the therapy and diagnosis of cancer, particularly lung cancer. As described further below, illustrative compositions of the present invention include, but are not restricted to, 15 polypeptides, particularly immunogenic polypeptides, polynucleotides encoding such polypeptides, antibodies and other binding agents, antigen presenting cells (APCs) and immune system cells (*e.g.*, T cells).

The practice of the present invention will employ, unless indicated specifically to the contrary, conventional methods of virology, immunology, microbiology, 20 molecular biology and recombinant DNA techniques within the skill of the art, many of which are described below for the purpose of illustration. Such techniques are explained fully in the literature. See, *e.g.*, Sambrook, et al. *Molecular Cloning: A Laboratory Manual* (2nd Edition, 1989); Maniatis et al. *Molecular Cloning: A Laboratory Manual* (1982); DNA Cloning: A Practical Approach, vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. 25 Gait, ed., 1984); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., 1985); Transcription and Translation (B. Hames & S. Higgins, eds., 1984); Animal Cell Culture (R. Freshney, ed., 1986); Perbal, *A Practical Guide to Molecular Cloning* (1984).

All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

As used in this specification and the appended claims, the singular forms "a," "an" and "the" include plural references unless the content clearly dictates otherwise.

## 5 Polypeptide Compositions

As used herein, the term "polypeptide" is used in its conventional meaning, *i.e.*, as a sequence of amino acids. The polypeptides are not limited to a specific length of the product; thus, peptides, oligopeptides, and proteins are included within the definition of polypeptide, and such terms may be used interchangeably herein unless specifically indicated otherwise. This term also does not refer to or exclude post-expression modifications of the polypeptide, for example, glycosylations, acetylations, phosphorylations and the like, as well as other modifications known in the art, both naturally occurring and non-naturally occurring. A polypeptide may be an entire protein, or a subsequence thereof. Particular polypeptides of interest in the context of this invention are amino acid subsequences comprising epitopes, *i.e.*, antigenic determinants substantially responsible for the immunogenic properties of a polypeptide and being capable of evoking an immune response.

Particularly illustrative polypeptides of the present invention comprise those encoded by a polynucleotide sequence set forth in any one of SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732, or a sequence that hybridizes under moderately stringent conditions, or, alternatively, under highly stringent conditions, to a polynucleotide sequence set forth in any one of SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732. Certain other illustrative polypeptides of the invention comprise amino acid sequences as set forth in any one of SEQ ID NOs: 391, 393, 395, 397, 421, 425-427, 434-439, 584-587 and .

The polypeptides of the present invention are sometimes herein referred to as lung tumor proteins or lung tumor polypeptides, as an indication that their identification has been based at least in part upon their increased levels of expression in lung tumor

samples. Thus, a "lung tumor polypeptide" or "lung tumor protein," refers generally to a polypeptide sequence of the present invention, or a polynucleotide sequence encoding such a polypeptide, that is expressed in a substantial proportion of lung tumor samples, for example preferably greater than about 20%, more preferably greater than about 30%, and most preferably greater than about 50% or more of lung tumor samples tested, at a level that is at least two fold, and preferably at least five fold, greater than the level of expression in normal tissues, as determined using a representative assay provided herein. A lung tumor polypeptide sequence of the invention, based upon its increased level of expression in tumor cells, has particular utility both as a diagnostic marker as well as a therapeutic target, as further described below.

In certain preferred embodiments, the polypeptides of the invention are immunogenic, *i.e.*, they react detectably within an immunoassay (such as an ELISA or T-cell stimulation assay) with antisera and/or T-cells from a patient with lung cancer. Screening for immunogenic activity can be performed using techniques well known to the skilled artisan. For example, such screens can be performed using methods such as those described in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In one illustrative example, a polypeptide may be immobilized on a solid support and contacted with patient sera to allow binding of antibodies within the sera to the immobilized polypeptide. Unbound sera may then be removed and bound antibodies detected using, for example,  $^{125}\text{I}$ -labeled Protein A.

As would be recognized by the skilled artisan, immunogenic portions of the polypeptides disclosed herein are also encompassed by the present invention. An "immunogenic portion," as used herein, is a fragment of an immunogenic polypeptide of the invention that itself is immunologically reactive (*i.e.*, specifically binds) with the B-cells and/or T-cell surface antigen receptors that recognize the polypeptide. Immunogenic portions may generally be identified using well known techniques, such as those summarized in Paul, *Fundamental Immunology*, 3rd ed., 243-247 (Raven Press, 1993) and references cited therein. Such techniques include screening polypeptides for the ability to react with antigen-specific antibodies, antisera and/or T-cell lines or clones. As used

herein, antisera and antibodies are "antigen-specific" if they specifically bind to an antigen (*i.e.*, they react with the protein in an ELISA or other immunoassay, and do not react detectably with unrelated proteins). Such antisera and antibodies may be prepared as described herein, and using well-known techniques.

5 In one preferred embodiment, an immunogenic portion of a polypeptide of the present invention is a portion that reacts with antisera and/or T-cells at a level that is not substantially less than the reactivity of the full-length polypeptide (*e.g.*, in an ELISA and/or T-cell reactivity assay). Preferably, the level of immunogenic activity of the immunogenic portion is at least about 50%, preferably at least about 70% and most preferably greater than about 90% of the immunogenicity for the full-length polypeptide. In some instances, 10 preferred immunogenic portions will be identified that have a level of immunogenic activity greater than that of the corresponding full-length polypeptide, *e.g.*, having greater than about 100% or 150% or more immunogenic activity.

In certain other embodiments, illustrative immunogenic portions may 15 include peptides in which an N-terminal leader sequence and/or transmembrane domain have been deleted. Other illustrative immunogenic portions will contain a small N- and/or C-terminal deletion (*e.g.*, 1-30 amino acids, preferably 5-15 amino acids), relative to the mature protein.

In another embodiment, a polypeptide composition of the invention may 20 also comprise one or more polypeptides that are immunologically reactive with T cells and/or antibodies generated against a polypeptide of the invention, particularly a polypeptide having an amino acid sequence disclosed herein, or to an immunogenic fragment or variant thereof.

In another embodiment of the invention, polypeptides are provided that 25 comprise one or more polypeptides that are capable of eliciting T cells and/or antibodies that are immunologically reactive with one or more polypeptides described herein, or one or more polypeptides encoded by contiguous nucleic acid sequences contained in the polynucleotide sequences disclosed herein, or immunogenic fragments or variants thereof,

or to one or more nucleic acid sequences which hybridize to one or more of these sequences under conditions of moderate to high stringency.

The present invention, in another aspect, provides polypeptide fragments comprising at least about 5, 10, 15, 20, 25, 50, or 100 contiguous amino acids, or more, including all intermediate lengths, of a polypeptide compositions set forth herein, such as those set forth in SEQ ID NOs: 391, 393, 395, 397, 421, 425-427, 434-439, 584-587 and , or those encoded by a polynucleotide sequence set forth in a sequence of SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732.

In another aspect, the present invention provides variants of the polypeptide compositions described herein. Polypeptide variants generally encompassed by the present invention will typically exhibit at least about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% or more identity (determined as described below), along its length, to a polypeptide sequences set forth herein.

In one preferred embodiment, the polypeptide fragments and variants provide by the present invention are immunologically reactive with an antibody and/or T-cell that reacts with a full-length polypeptide specifically set forth herein.

In another preferred embodiment, the polypeptide fragments and variants provided by the present invention exhibit a level of immunogenic activity of at least about 50%, preferably at least about 70%, and most preferably at least about 90% or more of that exhibited by a full-length polypeptide sequence specifically set forth herein.

A polypeptide "variant," as the term is used herein, is a polypeptide that typically differs from a polypeptide specifically disclosed herein in one or more substitutions, deletions, additions and/or insertions. Such variants may be naturally occurring or may be synthetically generated, for example, by modifying one or more of the above polypeptide sequences of the invention and evaluating their immunogenic activity as described herein and/or using any of a number of techniques well known in the art.

For example, certain illustrative variants of the polypeptides of the invention include those in which one or more portions, such as an N-terminal leader sequence or transmembrane domain, have been removed. Other illustrative variants include variants in

which a small portion (*e.g.*, 1-30 amino acids, preferably 5-15 amino acids) has been removed from the N- and/or C-terminal of the mature protein.

In many instances, a variant will contain conservative substitutions. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of peptide chemistry would expect the secondary structure and hydropathic nature of the polypeptide to be substantially unchanged. As described above, modifications may be made in the structure of the polynucleotides and polypeptides of the present invention and still obtain a functional molecule that encodes a variant or derivative polypeptide with desirable characteristics, *e.g.*, with immunogenic characteristics. When it is desired to alter the amino acid sequence of a polypeptide to create an equivalent, or even an improved, immunogenic variant or portion of a polypeptide of the invention, one skilled in the art will typically change one or more of the codons of the encoding DNA sequence according to Table 1.

For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence, and, of course, its underlying DNA coding sequence, and nevertheless obtain a protein with like properties. It is thus contemplated that various changes may be made in the peptide sequences of the disclosed compositions, or corresponding DNA sequences which encode said peptides without appreciable loss of their biological utility or activity.



TABLE 1

Amino Acids				Codons					
Alanine	Ala	A	GCA	GCC	GCG	GCU			
Cysteine	Cys	C	UGC	UGU					
Aspartic acid	Asp	D	GAC	GAU					
Glutamic acid	Glu	E	GAA	GAG					
Phenylalanine	Phe	F	UUC	UUU					
Glycine	Gly	G	GGA	GGC	GGG	GGU			
Histidine	His	H	CAC	CAU					
Isoleucine	Ile	I	AUA	AUC	AUU				
Lysine	Lys	K	AAA	AAG					
Leucine	Leu	L	UUA	UUG	CUA	CUC	CUG	CUU	
Methionine	Met	M	AUG						
Asparagine	Asn	N	AAC	AAU					
Proline	Pro	P	CCA	CCC	CCG	CCU			
Glutamine	Gln	Q	CAA	CAG					
Arginine	Arg	R	AGA	AGG	CGA	CGC	CGG	CGU	
Serine	Ser	S	AGC	AGU	UCA	UCC	UCG	UCU	
Threonine	Thr	T	ACA	ACC	ACG	ACU			
Valine	Val	V	GUA	GUC	GUG	GUU			
Tryptophan	Trp	W	UGG						
Tyrosine	Tyr	Y	UAC	UAU					

In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporated herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like. Each amino acid has been

assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics (Kyte and Doolittle, 1982). These values are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, *i.e.* still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within  $\pm 2$  is preferred, those within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred. It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U. S. Patent 4,554,101 (specifically incorporated herein by reference in its entirety), states that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

As detailed in U. S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0  $\pm$  1); glutamate (+3.0  $\pm$  1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5  $\pm$  1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4). It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In such changes, the substitution of amino acids whose hydrophilicity values are within  $\pm 2$  is preferred, those within  $\pm 1$  are particularly preferred, and those within  $\pm 0.5$  are even more particularly preferred.

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their

hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions that take various of the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

- 5 In addition, any polynucleotide may be further modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiesterase linkages in the backbone; and/or the inclusion of nontraditional bases such as inosine, queosine and wybutosine, as well as acetyl- methyl-, thio- and other
- 10 modified forms of adenine, cytidine, guanine, thymine and uridine.

- Amino acid substitutions may further be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids
- 15 with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine and valine; glycine and alanine; asparagine and glutamine; and serine, threonine, phenylalanine and tyrosine. Other groups of amino acids that may represent conservative changes include: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his. A variant may also, or
- 20 alternatively, contain nonconservative changes. In a preferred embodiment, variant polypeptides differ from a native sequence by substitution, deletion or addition of five amino acids or fewer. Variants may also (or alternatively) be modified by, for example, the deletion or addition of amino acids that have minimal influence on the immunogenicity, secondary structure and hydrophobic nature of the polypeptide.

- 25 As noted above, polypeptides may comprise a signal (or leader) sequence at the N-terminal end of the protein, which co-translationally or post-translationally directs transfer of the protein. The polypeptide may also be conjugated to a linker or other sequence for ease of synthesis, purification or identification of the polypeptide (e.g., poly-

His), or to enhance binding of the polypeptide to a solid support. For example, a polypeptide may be conjugated to an immunoglobulin Fc region.

When comparing polypeptide sequences, two sequences are said to be "identical" if the sequence of amino acids in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins – Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenies pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) *CABIOS* 5:151-153; Myers, E.W. and Muller W. (1988) *CABIOS* 4:11-17; Robinson, E.D. (1971) *Comb. Theor* 11:105; Santou, N. Nes, M. (1987) *Mol. Biol. Evol.* 4:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy – the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) *Proc. Natl. Acad., Sci. USA* 80:726-730.

Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity methods of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP,

BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nucl. Acids Res.* 25:3389-3402 and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. BLAST and BLAST 2.0 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides and polypeptides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. For amino acid sequences, a scoring matrix can be used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment.

In one preferred approach, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polypeptide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.*, the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

Within other illustrative embodiments, a polypeptide may be a fusion polypeptide that comprises multiple polypeptides as described herein, or that comprises at

least one polypeptide as described herein and an unrelated sequence, such as a known tumor protein. A fusion partner may, for example, assist in providing T helper epitopes (an immunological fusion partner), preferably T helper epitopes recognized by humans, or may assist in expressing the protein (an expression enhancer) at higher yields than the native recombinant protein. Certain preferred fusion partners are both immunological and expression enhancing fusion partners. Other fusion partners may be selected so as to increase the solubility of the polypeptide or to enable the polypeptide to be targeted to desired intracellular compartments. Still further fusion partners include affinity tags, which facilitate purification of the polypeptide.

Fusion polypeptides may generally be prepared using standard techniques, including chemical conjugation. Preferably, a fusion polypeptide is expressed as a recombinant polypeptide, allowing the production of increased levels, relative to a non-fused polypeptide, in an expression system. Briefly, DNA sequences encoding the polypeptide components may be assembled separately, and ligated into an appropriate expression vector. The 3' end of the DNA sequence encoding one polypeptide component is ligated, with or without a peptide linker, to the 5' end of a DNA sequence encoding the second polypeptide component so that the reading frames of the sequences are in phase. This permits translation into a single fusion polypeptide that retains the biological activity of both component polypeptides.

A peptide linker sequence may be employed to separate the first and second polypeptide components by a distance sufficient to ensure that each polypeptide folds into its secondary and tertiary structures. Such a peptide linker sequence is incorporated into the fusion polypeptide using standard techniques well known in the art. Suitable peptide linker sequences may be chosen based on the following factors: (1) their ability to adopt a flexible extended conformation; (2) their inability to adopt a secondary structure that could interact with functional epitopes on the first and second polypeptides; and (3) the lack of hydrophobic or charged residues that might react with the polypeptide functional epitopes. Preferred peptide linker sequences contain Gly, Asn and Ser residues. Other near neutral amino acids, such as Thr and Ala may also be used in the linker sequence. Amino acid

sequences which may be usefully employed as linkers include those disclosed in Maratea et al., *Gene* 40:39-46, 1985; Murphy et al., *Proc. Natl. Acad. Sci. USA* 83:8258-8262, 1986; U.S. Patent No. 4,935,233 and U.S. Patent No. 4,751,180. The linker sequence may generally be from 1 to about 50 amino acids in length. Linker sequences are not required  
 5 when the first and second polypeptides have non-essential N-terminal amino acid regions that can be used to separate the functional domains and prevent steric interference.

The ligated DNA sequences are operably linked to suitable transcriptional or translational regulatory elements. The regulatory elements responsible for expression of DNA are located only 5' to the DNA sequence encoding the first polypeptides. Similarly,  
 10 stop codons required to end translation and transcription termination signals are only present 3' to the DNA sequence encoding the second polypeptide.

The fusion polypeptide can comprise a polypeptide as described herein together with an unrelated immunogenic protein, such as an immunogenic protein capable of eliciting a recall response. Examples of such proteins include tetanus, tuberculosis and  
 15 hepatitis proteins (*see*, for example, Stoute et al. *New Engl. J. Med.*, 336:86-91, 1997).

In one preferred embodiment, the immunological fusion partner is derived from a *Mycobacterium* sp., such as a *Mycobacterium tuberculosis*-derived Ra12 fragment. Ra12 compositions and methods for their use in enhancing the expression and/or immunogenicity of heterologous polynucleotide/polypeptide sequences is described in U.S.  
 20 Patent Application 60/158,585, the disclosure of which is incorporated herein by reference in its entirety. Briefly, Ra12 refers to a polynucleotide region that is a subsequence of a *Mycobacterium tuberculosis* MTB32A nucleic acid. MTB32A is a serine protease of 32 KD molecular weight encoded by a gene in virulent and avirulent strains of *M. tuberculosis*. The nucleotide sequence and amino acid sequence of MTB32A have been  
 25 described (for example, U.S. Patent Application 60/158,585; *see also*, Skeiky et al., *Infection and Immun.* (1999) 67:3998-4007, incorporated herein by reference). C-terminal fragments of the MTB32A coding sequence express at high levels and remain as a soluble polypeptides throughout the purification process. Moreover, Ra12 may enhance the immunogenicity of heterologous immunogenic polypeptides with which it is fused. One

preferred Ra12 fusion polypeptide comprises a 14 KD C-terminal fragment corresponding to amino acid residues 192 to 323 of MTB32A. Other preferred Ra12 polynucleotides generally comprise at least about 15 consecutive nucleotides, at least about 30 nucleotides, at least about 60 nucleotides, at least about 100 nucleotides, at least about 200 nucleotides, 5 or at least about 300 nucleotides that encode a portion of a Ra12 polypeptide. Ra12 polynucleotides may comprise a native sequence (*i.e.*, an endogenous sequence that encodes a Ra12 polypeptide or a portion thereof) or may comprise a variant of such a sequence. Ra12 polynucleotide variants may contain one or more substitutions, additions, deletions and/or insertions such that the biological activity of the encoded fusion 10 polypeptide is not substantially diminished, relative to a fusion polypeptide comprising a native Ra12 polypeptide. Variants preferably exhibit at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity to a polynucleotide sequence that encodes a native Ra12 polypeptide or a portion thereof.

Within other preferred embodiments, an immunological fusion partner is 15 derived from protein D, a surface protein of the gram-negative bacterium *Haemophilus influenza B* (WO 91/18926). Preferably, a protein D derivative comprises approximately the first third of the protein (*e.g.*, the first N-terminal 100-110 amino acids), and a protein D derivative may be lipidated. Within certain preferred embodiments, the first 109 residues of a Lipoprotein D fusion partner is included on the N-terminus to provide the polypeptide 20 with additional exogenous T-cell epitopes and to increase the expression level in *E. coli* (thus functioning as an expression enhancer). The lipid tail ensures optimal presentation of the antigen to antigen presenting cells. Other fusion partners include the non-structural protein from influenzae virus, NS1 (hemagglutinin). Typically, the N-terminal 81 amino acids are used, although different fragments that include T-helper epitopes may be used.

25 In another embodiment, the immunological fusion partner is the protein known as LYTA, or a portion thereof (preferably a C-terminal portion). LYTA is derived from *Streptococcus pneumoniae*, which synthesizes an N-acetyl-L-alanine amidase known as amidase LYTA (encoded by the *LytA* gene; *Gene* 43:265-292, 1986). LYTA is an autolysin that specifically degrades certain bonds in the peptidoglycan backbone. The C-



terminal domain of the LYTA protein is responsible for the affinity to the choline or to some choline analogues such as DEAE. This property has been exploited for the development of *E. coli* C-LYTA expressing plasmids useful for expression of fusion proteins. Purification of hybrid proteins containing the C-LYTA fragment at the amino terminus has been described (see *Biotechnology* 10:795-798, 1992). Within a preferred embodiment, a repeat portion of LYTA may be incorporated into a fusion polypeptide. A repeat portion is found in the C-terminal region starting at residue 178. A particularly preferred repeat portion incorporates residues 188-305.

Yet another illustrative embodiment involves fusion polypeptides, and the polynucleotides encoding them, wherein the fusion partner comprises a targeting signal capable of directing a polypeptide to the endosomal/lysosomal compartment, as described in U.S. Patent No. 5,633,234. An immunogenic polypeptide of the invention, when fused with this targeting signal, will associate more efficiently with MHC class II molecules and thereby provide enhanced in vivo stimulation of CD4<sup>+</sup> T-cells specific for the polypeptide.

Polypeptides of the invention are prepared using any of a variety of well known synthetic and/or recombinant techniques, the latter of which are further described below. Polypeptides, portions and other variants generally less than about 150 amino acids can be generated by synthetic means, using techniques well known to those of ordinary skill in the art. In one illustrative example, such polypeptides are synthesized using any of the commercially available solid-phase techniques, such as the Merrifield solid-phase synthesis method, where amino acids are sequentially added to a growing amino acid chain. See Merrifield, *J. Am. Chem. Soc.* 85:2149-2146, 1963. Equipment for automated synthesis of polypeptides is commercially available from suppliers such as Perkin Elmer/Applied BioSystems Division (Foster City, CA), and may be operated according to the manufacturer's instructions.

In general, polypeptide compositions (including fusion polypeptides) of the invention are isolated. An "isolated" polypeptide is one that is removed from its original environment. For example, a naturally-occurring protein or polypeptide is isolated if it is separated from some or all of the coexisting materials in the natural system. Preferably,

such polypeptides are also purified, *e.g.*, are at least about 90% pure, more preferably at least about 95% pure and most preferably at least about 99% pure.

### Polynucleotide Compositions

5 The present invention, in other aspects, provides polynucleotide compositions. The terms "DNA" and "polynucleotide" are used essentially interchangeably herein to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. "Isolated," as used herein, means that a polynucleotide is substantially away from other coding sequences, and that the DNA molecule does not contain large portions of unrelated coding DNA, such as large chromosomal fragments or other  
10 functional genes or polypeptide coding regions. Of course, this refers to the DNA molecule as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

As will be understood by those skilled in the art, the polynucleotide compositions of this invention can include genomic sequences, extra-genomic and plasmid-  
15 encoded sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides, peptides and the like. Such segments may be naturally isolated, or modified synthetically by the hand of man.

As will be also recognized by the skilled artisan, polynucleotides of the invention may be single-stranded (coding or antisense) or double-stranded, and may be  
20 DNA (genomic, cDNA or synthetic) or RNA molecules. RNA molecules may include HnRNA molecules, which contain introns and correspond to a DNA molecule in a one-to-one manner, and mRNA molecules, which do not contain introns. Additional coding or non-coding sequences may, but need not, be present within a polynucleotide of the present invention, and a polynucleotide may, but need not, be linked to other molecules and/or  
25 support materials.

Polynucleotides may comprise a native sequence (*i.e.*, an endogenous sequence that encodes a polypeptide/protein of the invention or a portion thereof) or may

comprise a sequence that encodes a variant or derivative, preferably and immunogenic variant or derivative, of such a sequence.

Therefore, according to another aspect of the present invention, polynucleotide compositions are provided that comprise some or all of a polynucleotide sequence set forth in any one of SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732, complements of a polynucleotide sequence set forth in any one of SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732, and degenerate variants of a polynucleotide sequence set forth in any one of SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732. In certain preferred embodiments, the polynucleotide sequences set forth herein encode immunogenic polypeptides, as described above.

In other related embodiments, the present invention provides polynucleotide variants having substantial identity to the sequences disclosed herein in SEQ ID NOs: 217-390, 392, 394, 396, 398-420 422-424, 428-433, 440-583 and 588-732, for example those comprising at least 70% sequence identity, preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% or higher, sequence identity compared to a polynucleotide sequence of this invention using the methods described herein, (e.g., BLAST analysis using standard parameters, as described below). One skilled in this art will recognize that these values can be appropriately adjusted to determine corresponding identity of proteins encoded by two nucleotide sequences by taking into account codon degeneracy, amino acid similarity, reading frame positioning and the like.

Typically, polynucleotide variants will contain one or more substitutions, additions, deletions and/or insertions, preferably such that the immunogenicity of the polypeptide encoded by the variant polynucleotide is not substantially diminished relative to a polypeptide encoded by a polynucleotide sequence specifically set forth herein). The term "variants" should also be understood to encompass homologous genes of xenogenic origin.

In additional embodiments, the present invention provides polynucleotide fragments comprising various lengths of contiguous stretches of sequence identical to or

complementary to one or more of the sequences disclosed herein. For example, polynucleotides are provided by this invention that comprise at least about 10, 15, 20, 30, 40, 50, 75, 100, 150, 200, 300, 400, 500 or 1000 or more contiguous nucleotides of one or more of the sequences disclosed herein as well as all intermediate lengths there between. It will be readily understood that "intermediate lengths", in this context, means any length between the quoted values, such as 16, 17, 18, 19, *etc.*; 21, 22, 23, *etc.*; 30, 31, 32, *etc.*; 50, 51, 52, 53, *etc.*; 100, 101, 102, 103, *etc.*; 150, 151, 152, 153, *etc.*; including all integers through 200-500; 500-1,000, and the like.

In another embodiment of the invention, polynucleotide compositions are provided that are capable of hybridizing under moderate to high stringency conditions to a polynucleotide sequence provided herein, or a fragment thereof, or a complementary sequence thereof. Hybridization techniques are well known in the art of molecular biology. For purposes of illustration, suitable moderately stringent conditions for testing the hybridization of a polynucleotide of this invention with other polynucleotides include prewashing in a solution of 5 X SSC, 0.5% SDS, 1.0 mM EDTA (pH 8.0); hybridizing at 50°C-60°C, 5 X SSC, overnight; followed by washing twice at 65°C for 20 minutes with each of 2X, 0.5X and 0.2X SSC containing 0.1% SDS. One skilled in the art will understand that the stringency of hybridization can be readily manipulated, such as by altering the salt content of the hybridization solution and/or the temperature at which the hybridization is performed. For example, in another embodiment, suitable highly stringent hybridization conditions include those described above, with the exception that the temperature of hybridization is increased, *e.g.*, to 60-65°C or 65-70°C.

In certain preferred embodiments, the polynucleotides described above, *e.g.*, polynucleotide variants, fragments and hybridizing sequences, encode polypeptides that are immunologically cross-reactive with a polypeptide sequence specifically set forth herein. In other preferred embodiments, such polynucleotides encode polypeptides that have a level of immunogenic activity of at least about 50%, preferably at least about 70%, and more preferably at least about 90% of that for a polypeptide sequence specifically set forth herein.

The polynucleotides of the present invention, or fragments thereof, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, illustrative polynucleotide segments with total lengths of about 10,000, about 5000, about 3000, about 2,000, about 1,000, about 500, about 200, about 100, about 50 base pairs in length, and the like, (including all intermediate lengths) are contemplated to be useful in many implementations of this invention.

When comparing polynucleotide sequences, two sequences are said to be "identical" if the sequence of nucleotides in the two sequences is the same when aligned for maximum correspondence, as described below. Comparisons between two sequences are typically performed by comparing the sequences over a comparison window to identify and compare local regions of sequence similarity. A "comparison window" as used herein, refers to a segment of at least about 20 contiguous positions, usually 30 to about 75, 40 to about 50, in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Optimal alignment of sequences for comparison may be conducted using the Megalign program in the Lasergene suite of bioinformatics software (DNASTAR, Inc., Madison, WI), using default parameters. This program embodies several alignment schemes described in the following references: Dayhoff, M.O. (1978) A model of evolutionary change in proteins - Matrices for detecting distant relationships. In Dayhoff, M.O. (ed.) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, Washington DC Vol. 5, Suppl. 3, pp. 345-358; Hein J. (1990) Unified Approach to Alignment and Phylogenies pp. 626-645 *Methods in Enzymology* vol. 183, Academic Press, Inc., San Diego, CA; Higgins, D.G. and Sharp, P.M. (1989) *CABIOS* 5:151-153; Myers, E.W. and Muller W. (1988) *CABIOS* 4:11-17; Robinson, E.D. (1971)

*Comb. Theor* 11:105; Santou, N. Nes, M. (1987) *Mol. Biol. Evol.* 4:406-425; Sneath, P.H.A. and Sokal, R.R. (1973) *Numerical Taxonomy - the Principles and Practice of Numerical Taxonomy*, Freeman Press, San Francisco, CA; Wilbur, W.J. and Lipman, D.J. (1983) *Proc. Natl. Acad. Sci. USA* 80:726-730.

- 5 Alternatively, optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman (1981) *Add. APL. Math* 2:482, by the identity alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, by the search for similarity methods of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85: 2444, by computerized implementations of these algorithms (GAP, 10 BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Dr., Madison, WI), or by inspection.

One preferred example of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul et al. (1977) *Nucl. Acids Res.* 25:3389-3402 and Altschul et al. (1990) *J. Mol. Biol.* 215:403-410, respectively. BLAST and BLAST 2.0 15 can be used, for example with the parameters described herein, to determine percent sequence identity for the polynucleotides of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. In one illustrative example, cumulative scores can be calculated using, for 20 nucleotide sequences, the parameters M (reward score for a pair of matching residues; always >0) and N (penalty score for mismatching residues; always <0). Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of 25 either sequence is reached. The BLAST algorithm parameters W, T and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) *Proc. Natl. Acad. Sci. USA* 89:10915) alignments, (B) of 50, expectation (E) of 10, M=5, N=-4 and a comparison of both strands.

Preferably, the "percentage of sequence identity" is determined by comparing two optimally aligned sequences over a window of comparison of at least 20 positions, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (*i.e.*, gaps) of 20 percent or less, usually 5 to 15 percent, or 10 to 12 percent, as compared to the reference sequences (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid bases occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the reference sequence (*i.e.*, the window size) and multiplying the results by 100 to yield the percentage of sequence identity.

It will be appreciated by those of ordinary skill in the art that, as a result of the degeneracy of the genetic code, there are many nucleotide sequences that encode a polypeptide as described herein. Some of these polynucleotides bear minimal homology to the nucleotide sequence of any native gene. Nonetheless, polynucleotides that vary due to differences in codon usage are specifically contemplated by the present invention. Further, alleles of the genes comprising the polynucleotide sequences provided herein are within the scope of the present invention. Alleles are endogenous genes that are altered as a result of one or more mutations, such as deletions, additions and/or substitutions of nucleotides. The resulting mRNA and protein may, but need not, have an altered structure or function. Alleles may be identified using standard techniques (such as hybridization, amplification and/or database sequence comparison).

Therefore, in another embodiment of the invention, a mutagenesis approach, such as site-specific mutagenesis, is employed for the preparation of immunogenic variants and/or derivatives of the polypeptides described herein. By this approach, specific modifications in a polypeptide sequence can be made through mutagenesis of the underlying polynucleotides that encode them. These techniques provides a straightforward approach to prepare and test sequence variants, for example, incorporating one or more of

the foregoing considerations, by introducing one or more nucleotide sequence changes into the polynucleotide.

Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Mutations may be employed in a selected polynucleotide sequence to improve, alter, decrease, modify, or otherwise change the properties of the polynucleotide itself, and/or alter the properties, activity, composition, stability, or primary sequence of the encoded polypeptide.

In certain embodiments of the present invention, the inventors contemplate the mutagenesis of the disclosed polynucleotide sequences to alter one or more properties of the encoded polypeptide, such as the immunogenicity of a polypeptide vaccine. The techniques of site-specific mutagenesis are well known in the art, and are widely used to create variants of both polypeptides and polynucleotides. For example, site-specific mutagenesis is often used to alter a specific portion of a DNA molecule. In such embodiments, a primer comprising typically about 14 to about 25 nucleotides or so in length is employed, with about 5 to about 10 residues on both sides of the junction of the sequence being altered.

As will be appreciated by those of skill in the art, site-specific mutagenesis techniques have often employed a phage vector that exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially-available and their use is generally well-known to those skilled in the art. Double-stranded plasmids are also routinely employed in site directed mutagenesis that eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a double-stranded vector that includes within its sequence a DNA sequence that encodes the desired peptide.



An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically. This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

The preparation of sequence variants of the selected peptide-encoding DNA segments using site-directed mutagenesis provides a means of producing potentially useful species and is not meant to be limiting as there are other ways in which sequence variants of peptides and the DNA sequences encoding them may be obtained. For example, recombinant vectors encoding the desired peptide sequence may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants. Specific details regarding these methods and protocols are found in the teachings of Maloy *et al.*, 1994; Segal, 1976; Prokop and Bajpai, 1991; Kuby, 1994; and Maniatis *et al.*, 1982, each incorporated herein by reference, for that purpose.

As used herein, the term "oligonucleotide directed mutagenesis procedure" refers to template-dependent processes and vector-mediated propagation which result in an increase in the concentration of a specific nucleic acid molecule relative to its initial concentration, or in an increase in the concentration of a detectable signal, such as amplification. As used herein, the term "oligonucleotide directed mutagenesis procedure" is intended to refer to a process that involves the template-dependent extension of a primer molecule. The term template dependent process refers to nucleic acid synthesis of an RNA or a DNA molecule wherein the sequence of the newly synthesized strand of nucleic acid is dictated by the well-known rules of complementary base pairing (see, for example, Watson, 1987). Typically, vector mediated methodologies involve the introduction of the nucleic acid fragment into a DNA or RNA vector, the clonal amplification of the vector, and the recovery of the amplified nucleic acid fragment. Examples of such methodologies are

provided by U. S. Patent No. 4,237,224, specifically incorporated herein by reference in its entirety.

In another approach for the production of polypeptide variants of the present invention, recursive sequence recombination, as described in U.S. Patent No. 5,837,458, may be employed. In this approach, iterative cycles of recombination and screening or selection are performed to "evolve" individual polynucleotide variants of the invention having, for example, enhanced immunogenic activity.

In other embodiments of the present invention, the polynucleotide sequences provided herein can be advantageously used as probes or primers for nucleic acid hybridization. As such, it is contemplated that nucleic acid segments that comprise a sequence region of at least about 15 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 15 nucleotide long contiguous sequence disclosed herein will find particular utility. Longer contiguous identical or complementary sequences, *e.g.*, those of about 20, 30, 40, 50, 100, 200, 500, 1000 (including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

The ability of such nucleic acid probes to specifically hybridize to a sequence of interest will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are also envisioned, such as the use of the sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

Polynucleotide molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so (including intermediate lengths as well), identical or complementary to a polynucleotide sequence disclosed herein, are particularly contemplated as hybridization probes for use in, *e.g.*, Southern and Northern blotting. This would allow a gene product, or fragment thereof, to be analyzed, both in diverse cell types and also in various bacterial cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of the particular nucleic acid segment. Smaller

fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 15 and about 100 nucleotides, but larger contiguous complementarity stretches may be used, according to the length complementary sequences one wishes to detect.

5           The use of a hybridization probe of about 15-25 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 15 bases in length are generally preferred, though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will  
10       generally prefer to design nucleic acid molecules having gene-complementary stretches of 15 to 25 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All that is required is to review the sequences set forth herein, or to any continuous portion of the sequences, from about 15-25 nucleotides in length up to  
15       and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and primer sequences may be governed by various factors. For example, one may wish to employ primers from towards the termini of the total sequence.

Small polynucleotide segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced  
20       using an automated oligonucleotide synthesizer. Also, fragments may be obtained by application of nucleic acid reproduction technology, such as the PCR™ technology of U. S. Patent 4,683,202 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA techniques generally known to those of skill in the art of molecular biology.

25           The nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of the entire gene or gene fragments of interest. Depending on the application envisioned, one will typically desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will

typically desire to employ relatively stringent conditions to form the hybrids, *e.g.*, one will select relatively low salt and/or high temperature conditions, such as provided by a salt concentration of from about 0.02 M to about 0.15 M salt at temperatures of from about 50°C to about 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating related sequences.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template, less stringent (reduced stringency) hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ salt conditions such as those of from about 0.15 M to about 0.9 M salt, at temperatures ranging from about 20°C to about 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

According to another embodiment of the present invention, polynucleotide compositions comprising antisense oligonucleotides are provided. Antisense oligonucleotides have been demonstrated to be effective and targeted inhibitors of protein synthesis, and, consequently, provide a therapeutic approach by which a disease can be treated by inhibiting the synthesis of proteins that contribute to the disease. The efficacy of antisense oligonucleotides for inhibiting protein synthesis is well established. For example, the synthesis of polygalacturonase and the muscarine type 2 acetylcholine receptor are inhibited by antisense oligonucleotides directed to their respective mRNA sequences (U. S. Patent 5,739,119 and U. S. Patent 5,759,829). Further, examples of antisense inhibition have been demonstrated with the nuclear protein cyclin, the multiple drug resistance gene (MDG1), ICAM-1, E-selectin, STK-1, striatal GABA<sub>A</sub> receptor and human EGF (Jaskulski

*et al.*, Science. 1988 Jun 10;240(4858):1544-6; Vasanthakumar and Ahmed, Cancer Commun. 1989;1(4):225-32; Peris *et al.*, Brain Res Mol Brain Res. 1998 Jun 15;57(2):310-20; U. S. Patent 5,801,154; U.S. Patent 5,789,573; U. S. Patent 5,718,709 and U.S. Patent 5,610,288). Antisense constructs have also been described that inhibit and can be used to  
 5 treat a variety of abnormal cellular proliferations, *e.g.* cancer (U. S. Patent 5,747,470; U. S. Patent 5,591,317 and U. S. Patent 5,783,683).

Therefore, in certain embodiments, the present invention provides oligonucleotide sequences that comprise all, or a portion of, any sequence that is capable of specifically binding to polynucleotide sequence described herein, or a complement thereof.

10 In one embodiment, the antisense oligonucleotides comprise DNA or derivatives thereof. In another embodiment, the oligonucleotides comprise RNA or derivatives thereof. In a third embodiment, the oligonucleotides are modified DNAs comprising a phosphorothioated modified backbone. In a fourth embodiment, the oligonucleotide sequences comprise peptide nucleic acids or derivatives thereof. In each case, preferred  
 15 compositions comprise a sequence region that is complementary, and more preferably substantially-complementary, and even more preferably, completely complementary to one or more portions of polynucleotides disclosed herein. Selection of antisense compositions specific for a given gene sequence is based upon analysis of the chosen target sequence and determination of secondary structure,  $T_m$ , binding energy, and relative stability. Antisense  
 20 compositions may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to the target mRNA in a host cell. Highly preferred target regions of the mRNA, are those which are at or near the AUG translation initiation codon, and those sequences which are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target  
 25 site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software and/or the BLASTN 2.0.5 algorithm software (Altschul *et al.*, Nucleic Acids Res. 1997, 25(17):3389-402).

The use of an antisense delivery method employing a short peptide vector, termed MPG (27 residues), is also contemplated. The MPG peptide contains a

hydrophobic domain derived from the fusion sequence of HIV gp41 and a hydrophilic domain from the nuclear localization sequence of SV40 T-antigen (Morris *et al.*, Nucleic Acids Res. 1997 Jul 15;25(14):2730-6). It has been demonstrated that several molecules of the MPG peptide coat the antisense oligonucleotides and can be delivered into cultured  
 5 mammalian cells in less than 1 hour with relatively high efficiency (90%). Further, the interaction with MPG strongly increases both the stability of the oligonucleotide to nuclease and the ability to cross the plasma membrane.

According to another embodiment of the invention, the polynucleotide compositions described herein are used in the design and preparation of ribozyme  
 10 molecules for inhibiting expression of the tumor polypeptides and proteins of the present invention in tumor cells. Ribozymes are RNA-protein complexes that cleave nucleic acids in a site-specific fashion. Ribozymes have specific catalytic domains that possess endonuclease activity (Kim and Cech, Proc Natl Acad Sci U S A. 1987 Dec;84(24):8788-92; Forster and Symons, Cell. 1987 Apr 24;49(2):211-20). For example, a large number of  
 15 ribozymes accelerate phosphoester transfer reactions with a high degree of specificity, often cleaving only one of several phosphoesters in an oligonucleotide substrate (Cech *et al.*, Cell. 1981 Dec;27(3 Pt 2):487-96; Michel and Westhof, J Mol Biol. 1990 Dec 5;216(3):585-610; Reinhold-Hurek and Shub, Nature. 1992 May 14;357(6374):173-6). This specificity has been attributed to the requirement that the substrate bind via specific  
 20 base-pairing interactions to the internal guide sequence ("IGS") of the ribozyme prior to chemical reaction.

Six basic varieties of naturally occurring enzymatic RNAs are known presently. Each can catalyze the hydrolysis of RNA phosphodiester bonds *in trans* (and thus can cleave other RNA molecules) under physiological conditions. In general,  
 25 enzymatic nucleic acids act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base-pairing, and once bound to the correct site, acts enzymatically to cut the target RNA.

Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

5 The enzymatic nature of a ribozyme is advantageous over many technologies, such as antisense technology (where a nucleic acid molecule simply binds to a nucleic acid target to block its translation) since the concentration of ribozyme necessary to affect a therapeutic treatment is lower than that of an antisense oligonucleotide. This advantage reflects the ability of the ribozyme to act enzymatically. Thus, a single ribozyme molecule is able to cleave many molecules of target RNA. In addition, the ribozyme is a highly specific inhibitor, with the specificity of inhibition depending not only on the base pairing mechanism of binding to the target RNA, but also on the mechanism of target RNA cleavage. Single mismatches, or base-substitutions, near the site of cleavage can completely eliminate catalytic activity of a ribozyme. Similar mismatches in antisense molecules do not prevent their action (Woolf *et al.*, Proc Natl Acad Sci U S A. 1992 Aug 15;89(16):7305-9). Thus, the specificity of action of a ribozyme is greater than that of an antisense oligonucleotide binding the same RNA site.

The enzymatic nucleic acid molecule may be formed in a hammerhead, hairpin, a hepatitis  $\delta$  virus, group I intron or RNaseP RNA (in association with an RNA guide sequence) or Neurospora VS RNA motif. Examples of hammerhead motifs are described by Rossi *et al.* Nucleic Acids Res. 1992 Sep 11;20(17):4559-65. Examples of hairpin motifs are described by Hampel *et al.* (Eur. Pat. Appl. Publ. No. EP 0360257), Hampel and Tritz, Biochemistry 1989 Jun 13;28(12):4929-33; Hampel *et al.*, Nucleic Acids Res. 1990 Jan 25;18(2):299-304 and U. S. Patent 5,631,359. An example of the hepatitis  $\delta$  virus motif is described by Perrotta and Been, Biochemistry. 1992 Dec 25 1;31(47):11843-52; an example of the RNaseP motif is described by Guerrier-Takada *et al.*, Cell. 1983 Dec;35(3 Pt 2):849-57; Neurospora VS RNA ribozyme motif is described by Collins (Saville and Collins, Cell. 1990 May 18;61(4):685-96; Saville and Collins, Proc Natl Acad Sci U S A. 1991 Oct 1;88(19):8826-30; Collins and Olive, Biochemistry. 1993

Mar 23;32(11):2795-9); and an example of the Group I intron is described in (U. S. Patent 4,987,071). All that is important in an enzymatic nucleic acid molecule of this invention is that it has a specific substrate binding site which is complementary to one or more of the target gene RNA regions, and that it have nucleotide sequences within or surrounding that substrate binding site which impart an RNA cleaving activity to the molecule. Thus the ribozyme constructs need not be limited to specific motifs mentioned herein.

Ribozymes may be designed as described in Int. Pat. Appl. Publ. No. WO 93/23569 and Int. Pat. Appl. Publ. No. WO 94/02595, each specifically incorporated herein by reference) and synthesized to be tested *in vitro* and *in vivo*, as described. Such ribozymes can also be optimized for delivery. While specific examples are provided, those in the art will recognize that equivalent RNA targets in other species can be utilized when necessary.

Ribozyme activity can be optimized by altering the length of the ribozyme binding arms, or chemically synthesizing ribozymes with modifications that prevent their degradation by serum ribonucleases (see *e.g.*, Int. Pat. Appl. Publ. No. WO 92/07065; Int. Pat. Appl. Publ. No. WO 93/15187; Int. Pat. Appl. Publ. No. WO 91/03162; Eur. Pat. Appl. Publ. No. 92110298.4; U. S. Patent 5,334,711; and Int. Pat. Appl. Publ. No. WO 94/13688, which describe various chemical modifications that can be made to the sugar moieties of enzymatic RNA molecules), modifications which enhance their efficacy in cells, and removal of stem II bases to shorten RNA synthesis times and reduce chemical requirements.

Sullivan *et al.* (Int. Pat. Appl. Publ. No. WO 94/02595) describes the general methods for delivery of enzymatic RNA molecules. Ribozymes may be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. For some indications, ribozymes may be directly delivered *ex vivo* to cells or tissues with or without the aforementioned vehicles. Alternatively, the RNA/vehicle combination may be locally delivered by direct inhalation, by direct injection



or by use of a catheter, infusion pump or stent. Other routes of delivery include, but are not limited to, intravascular, intramuscular, subcutaneous or joint injection, aerosol inhalation, oral (tablet or pill form), topical, systemic, ocular, intraperitoneal and/or intrathecal delivery. More detailed descriptions of ribozyme delivery and administration are provided in Int. Pat. Appl. Publ. No. WO 94/02595 and Int. Pat. Appl. Publ. No. WO 93/23569, each specifically incorporated herein by reference.

Another means of accumulating high concentrations of a ribozyme(s) within cells is to incorporate the ribozyme-encoding sequences into a DNA expression vector. Transcription of the ribozyme sequences are driven from a promoter for eukaryotic RNA polymerase I (pol I), RNA polymerase II (pol II), or RNA polymerase III (pol III). Transcripts from pol II or pol III promoters will be expressed at high levels in all cells; the levels of a given pol II promoter in a given cell type will depend on the nature of the gene regulatory sequences (enhancers, silencers, *etc.*) present nearby. Prokaryotic RNA polymerase promoters may also be used, providing that the prokaryotic RNA polymerase enzyme is expressed in the appropriate cells. Ribozymes expressed from such promoters have been shown to function in mammalian cells. Such transcription units can be incorporated into a variety of vectors for introduction into mammalian cells, including but not restricted to, plasmid DNA vectors, viral DNA vectors (such as adenovirus or adeno-associated vectors), or viral RNA vectors (such as retroviral, semliki forest virus, sindbis virus vectors).

In another embodiment of the invention, peptide nucleic acids (PNAs) compositions are provided. PNA is a DNA mimic in which the nucleobases are attached to a pseudopeptide backbone (Good and Nielsen, *Antisense Nucleic Acid Drug Dev.* 1997 7(4) 431-37). PNA is able to be utilized in a number of methods that traditionally have used RNA or DNA. Often PNA sequences perform better in techniques than the corresponding RNA or DNA sequences and have utilities that are not inherent to RNA or DNA. A review of PNA including methods of making, characteristics of, and methods of using, is provided by Corey (*Trends Biotechnol* 1997 Jun;15(6):224-9). As such, in certain embodiments, one may prepare PNA sequences that are complementary to one or more portions of the ACE

mRNA sequence, and such PNA compositions may be used to regulate, alter, decrease, or reduce the translation of ACE-specific mRNA, and thereby alter the level of ACE activity in a host cell to which such PNA compositions have been administered.

- PNAs have 2-aminoethyl-glycine linkages replacing the normal phosphodiester backbone of DNA (Nielsen *et al.*, *Science* 1991 Dec 6;254(5037):1497-500; Hanvey *et al.*, *Science*. 1992 Nov 27;258(5087):1481-5; Hyrup and Nielsen, *Bioorg Med Chem.* 1996 Jan;4(1):5-23). This chemistry has three important consequences: firstly, in contrast to DNA or phosphorothioate oligonucleotides, PNAs are neutral molecules; secondly, PNAs are achiral, which avoids the need to develop a stereoselective synthesis; and thirdly, PNA synthesis uses standard Boc or Fmoc protocols for solid-phase peptide synthesis, although other methods, including a modified Merrifield method, have been used.

- PNA monomers or ready-made oligomers are commercially available from PerSeptive Biosystems (Framingham, MA). PNA syntheses by either Boc or Fmoc protocols are straightforward using manual or automated protocols (Norton *et al.*, *Bioorg Med Chem.* 1995 Apr;3(4):437-45). The manual protocol lends itself to the production of chemically modified PNAs or the simultaneous synthesis of families of closely related PNAs.

- As with peptide synthesis, the success of a particular PNA synthesis will depend on the properties of the chosen sequence. For example, while in theory PNAs can incorporate any combination of nucleotide bases, the presence of adjacent purines can lead to deletions of one or more residues in the product. In expectation of this difficulty, it is suggested that, in producing PNAs with adjacent purines, one should repeat the coupling of residues likely to be added inefficiently. This should be followed by the purification of PNAs by reverse-phase high-pressure liquid chromatography, providing yields and purity of product similar to those observed during the synthesis of peptides.

Modifications of PNAs for a given application may be accomplished by coupling amino acids during solid-phase synthesis or by attaching compounds that contain a carboxylic acid group to the exposed N-terminal amine. Alternatively, PNAs can be

modified after synthesis by coupling to an introduced lysine or cysteine. The ease with which PNAs can be modified facilitates optimization for better solubility or for specific functional requirements. Once synthesized, the identity of PNAs and their derivatives can be confirmed by mass spectrometry. Several studies have made and utilized modifications of PNAs (for example, Norton *et al.*, Bioorg Med Chem. 1995 Apr;3(4):437-45; Petersen *et al.*, J Pept Sci. 1995 May-Jun;1(3):175-83; Orum *et al.*, Biotechniques. 1995 Sep;19(3):472-80; Footer *et al.*, Biochemistry. 1996 Aug 20;35(33):10673-9; Griffith *et al.*, Nucleic Acids Res. 1995 Aug 11;23(15):3003-8; Pardridge *et al.*, Proc Natl Acad Sci U S A. 1995 Jun 6;92(12):5592-6; Boffa *et al.*, Proc Natl Acad Sci U S A. 1995 Mar 14;92(6):1901-5; Gambacorti-Passerini *et al.*, Blood. 1996 Aug 15;88(4):1411-7; Armitage *et al.*, Proc Natl Acad Sci U S A. 1997 Nov 11;94(23):12320-5; Seeger *et al.*, Biotechniques. 1997 Sep;23(3):512-7). U.S. Patent No. 5,700,922 discusses PNA-DNA-PNA chimeric molecules and their uses in diagnostics, modulating protein in organisms, and treatment of conditions susceptible to therapeutics.

Methods of characterizing the antisense binding properties of PNAs are discussed in Rose (Anal Chem. 1993 Dec 15;65(24):3545-9) and Jensen *et al.* (Biochemistry. 1997 Apr 22;36(16):5072-7). Rose uses capillary gel electrophoresis to determine binding of PNAs to their complementary oligonucleotide, measuring the relative binding kinetics and stoichiometry. Similar types of measurements were made by Jensen *et al.* using BIAcore™ technology.

Other applications of PNAs that have been described and will be apparent to the skilled artisan include use in DNA strand invasion, antisense inhibition, mutational analysis, enhancers of transcription, nucleic acid purification, isolation of transcriptionally active genes, blocking of transcription factor binding, genome cleavage, biosensors, *in situ* hybridization, and the like.

#### Polynucleotide Identification, Characterization and Expression

Polynucleotides compositions of the present invention may be identified, prepared and/or manipulated using any of a variety of well established techniques (see

generally, Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989, and other like references). For example, a polynucleotide may be identified, as described in more detail below, by screening a microarray of cDNAs for tumor-associated expression (*i.e.*, expression that is at least two fold greater in a tumor than in normal tissue, as determined using a representative assay provided herein). Such screens may be performed, for example, using the microarray technology of Affymetrix, Inc. (Santa Clara, CA) according to the manufacturer's instructions (and essentially as described by Schena et al., *Proc. Natl. Acad. Sci. USA* 93:10614-10619, 1996 and Heller et al., *Proc. Natl. Acad. Sci. USA* 94:2150-2155, 1997).

10 Alternatively, polynucleotides may be amplified from cDNA prepared from cells expressing the proteins described herein, such as tumor cells.

Many template dependent processes are available to amplify a target sequences of interest present in a sample. One of the best known amplification methods is the polymerase chain reaction (PCR<sup>TM</sup>) which is described in detail in U.S. Patent Nos. 15 4,683,195, 4,683,202 and 4,800,159, each of which is incorporated herein by reference in its entirety. Briefly, in PCR<sup>TM</sup>, two primer sequences are prepared which are complementary to regions on opposite complementary strands of the target sequence. An excess of deoxynucleoside triphosphates is added to a reaction mixture along with a DNA polymerase (*e.g.*, Taq polymerase). If the target sequence is present in a sample, the 20 primers will bind to the target and the polymerase will cause the primers to be extended along the target sequence by adding on nucleotides. By raising and lowering the temperature of the reaction mixture, the extended primers will dissociate from the target to form reaction products, excess primers will bind to the target and to the reaction product and the process is repeated. Preferably reverse transcription and PCR<sup>TM</sup> amplification 25 procedure may be performed in order to quantify the amount of mRNA amplified. Polymerase chain reaction methodologies are well known in the art.

Any of a number of other template dependent processes, many of which are variations of the PCR<sup>TM</sup> amplification technique, are readily known and available in the art. Illustratively, some such methods include the ligase chain reaction (referred to as

LCR), described, for example, in Eur. Pat. Appl. Publ. No. 320,308 and U.S. Patent No. 4,883,750; Qbeta Replicase, described in PCT Intl. Pat. Appl. Publ. No. PCT/US87/00880; Strand Displacement Amplification (SDA) and Repair Chain Reaction (RCR). Still other amplification methods are described in Great Britain Pat. Appl. No. 2 202 328, and in PCT Intl. Pat. Appl. Publ. No. PCT/US89/01025. Other nucleic acid amplification procedures include transcription-based amplification systems (TAS) (PCT Intl. Pat. Appl. Publ. No. WO 88/10315), including nucleic acid sequence based amplification (NASBA) and 3SR. Eur. Pat. Appl. Publ. No. 329,822 describes a nucleic acid amplification process involving cyclically synthesizing single-stranded RNA ("ssRNA"), ssDNA, and double-stranded DNA (dsDNA). PCT Intl. Pat. Appl. Publ. No. WO 89/06700 describes a nucleic acid sequence amplification scheme based on the hybridization of a promoter/primer sequence to a target single-stranded DNA ("ssDNA") followed by transcription of many RNA copies of the sequence. Other amplification methods such as "RACE" (Frohman, 1990), and "one-sided PCR" (Ohara, 1989) are also well-known to those of skill in the art.

15 An amplified portion of a polynucleotide of the present invention may be used to isolate a full length gene from a suitable library (e.g., a tumor cDNA library) using well known techniques. Within such techniques, a library (cDNA or genomic) is screened using one or more polynucleotide probes or primers suitable for amplification. Preferably, a library is size-selected to include larger molecules. Random primed libraries may also be preferred for identifying 5' and upstream regions of genes. Genomic libraries are preferred for obtaining introns and extending 5' sequences.

25 For hybridization techniques, a partial sequence may be labeled (e.g., by nick-translation or end-labeling with  $^{32}\text{P}$ ) using well-known techniques. A bacterial or bacteriophage library is then generally screened by hybridizing filters containing denatured bacterial colonies (or lawns containing phage plaques) with the labeled probe (see Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989). Hybridizing colonies or plaques are selected and expanded, and the DNA is isolated for further analysis. cDNA clones may be analyzed to determine the amount of additional sequence by, for example, PCR using a primer from

the partial sequence and a primer from the vector. Restriction maps and partial sequences may be generated to identify one or more overlapping clones. The complete sequence may then be determined using standard techniques, which may involve generating a series of deletion clones. The resulting overlapping sequences can then be assembled into a single contiguous sequence. A full length cDNA molecule can be generated by ligating suitable fragments, using well known techniques.

Alternatively, amplification techniques, such as those described above, can be useful for obtaining a full length coding sequence from a partial cDNA sequence. One such amplification technique is inverse PCR (see Triglia et al., *Nucl. Acids Res.* 16:8186, 1988), which uses restriction enzymes to generate a fragment in the known region of the gene. The fragment is then circularized by intramolecular ligation and used as a template for PCR with divergent primers derived from the known region. Within an alternative approach, sequences adjacent to a partial sequence may be retrieved by amplification with a primer to a linker sequence and a primer specific to a known region. The amplified sequences are typically subjected to a second round of amplification with the same linker primer and a second primer specific to the known region. A variation on this procedure, which employs two primers that initiate extension in opposite directions from the known sequence, is described in WO 96/38591. Another such technique is known as "rapid amplification of cDNA ends" or RACE. This technique involves the use of an internal primer and an external primer, which hybridizes to a polyA region or vector sequence, to identify sequences that are 5' and 3' of a known sequence. Additional techniques include capture PCR (Lagerstrom et al., *PCR Methods Applic.* 1:111-19, 1991) and walking PCR (Parker et al., *Nucl. Acids. Res.* 19:3055-60, 1991). Other methods employing amplification may also be employed to obtain a full length cDNA sequence.

In certain instances, it is possible to obtain a full length cDNA sequence by analysis of sequences provided in an expressed sequence tag (EST) database, such as that available from GenBank. Searches for overlapping ESTs may generally be performed using well known programs (e.g., NCBI BLAST searches), and such ESTs may be used to

generate a contiguous full length sequence. Full length DNA sequences may also be obtained by analysis of genomic fragments.

In other embodiments of the invention, polynucleotide sequences or fragments thereof which encode polypeptides of the invention, or fusion proteins or functional equivalents thereof, may be used in recombinant DNA molecules to direct expression of a polypeptide in appropriate host cells. Due to the inherent degeneracy of the genetic code, other DNA sequences that encode substantially the same or a functionally equivalent amino acid sequence may be produced and these sequences may be used to clone and express a given polypeptide.

As will be understood by those of skill in the art, it may be advantageous in some instances to produce polypeptide-encoding nucleotide sequences possessing non-naturally occurring codons. For example, codons preferred by a particular prokaryotic or eukaryotic host can be selected to increase the rate of protein expression or to produce a recombinant RNA transcript having desirable properties, such as a half-life which is longer than that of a transcript generated from the naturally occurring sequence.

Moreover, the polynucleotide sequences of the present invention can be engineered using methods generally known in the art in order to alter polypeptide encoding sequences for a variety of reasons, including but not limited to, alterations which modify the cloning, processing, and/or expression of the gene product. For example, DNA shuffling by random fragmentation and PCR reassembly of gene fragments and synthetic oligonucleotides may be used to engineer the nucleotide sequences. In addition, site-directed mutagenesis may be used to insert new restriction sites, alter glycosylation patterns, change codon preference, produce splice variants, or introduce mutations, and so forth.

In another embodiment of the invention, natural, modified, or recombinant nucleic acid sequences may be ligated to a heterologous sequence to encode a fusion protein. For example, to screen peptide libraries for inhibitors of polypeptide activity, it may be useful to encode a chimeric protein that can be recognized by a commercially available antibody. A fusion protein may also be engineered to contain a cleavage site

located between the polypeptide-encoding sequence and the heterologous protein sequence, so that the polypeptide may be cleaved and purified away from the heterologous moiety.

- Sequences encoding a desired polypeptide may be synthesized, in whole or in part, using chemical methods well known in the art (see Caruthers, M. H. et al. (1980) *Nucl. Acids Res. Symp. Ser.* 215-223, Horn, T. et al. (1980) *Nucl. Acids Res. Symp. Ser.* 225-232). Alternatively, the protein itself may be produced using chemical methods to synthesize the amino acid sequence of a polypeptide, or a portion thereof. For example, peptide synthesis can be performed using various solid-phase techniques (Roberge, J. Y. et al. (1995) *Science* 269:202-204) and automated synthesis may be achieved, for example, using the ABI 431A Peptide Synthesizer (Perkin Elmer, Palo Alto, CA).

- A newly synthesized peptide may be substantially purified by preparative high performance liquid chromatography (e.g., Creighton, T. (1983) *Proteins, Structures and Molecular Principles*, WH Freeman and Co., New York, N.Y.) or other comparable techniques available in the art. The composition of the synthetic peptides may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure). Additionally, the amino acid sequence of a polypeptide, or any part thereof, may be altered during direct synthesis and/or combined using chemical methods with sequences from other proteins, or any part thereof, to produce a variant polypeptide.

- In order to express a desired polypeptide, the nucleotide sequences encoding the polypeptide, or functional equivalents, may be inserted into appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Methods which are well known to those skilled in the art may be used to construct expression vectors containing sequences encoding a polypeptide of interest and appropriate transcriptional and translational control elements. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination. Such techniques are described, for example, in Sambrook, J. et al. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, Plainview, N.Y., and Ausubel, F. M. et al. (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.



A variety of expression vector/host systems may be utilized to contain and express polynucleotide sequences. These include, but are not limited to, microorganisms such as bacteria transformed with recombinant bacteriophage, plasmid, or cosmid DNA expression vectors; yeast transformed with yeast expression vectors; insect cell systems  
5 infected with virus expression vectors (e.g., baculovirus); plant cell systems transformed with virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or with bacterial expression vectors (e.g., Ti or pBR322 plasmids); or animal cell systems.

The "control elements" or "regulatory sequences" present in an expression  
10 vector are those non-translated regions of the vector--enhancers, promoters, 5' and 3' untranslated regions--which interact with host cellular proteins to carry out transcription and translation. Such elements may vary in their strength and specificity. Depending on the vector system and host utilized, any number of suitable transcription and translation elements, including constitutive and inducible promoters, may be used. For example, when  
15 cloning in bacterial systems, inducible promoters such as the hybrid lacZ promoter of the PBLUESCRIPT phagemid (Stratagene, La Jolla, Calif.) or PSPO11 plasmid (Gibco BRL, Gaithersburg, MD) and the like may be used. In mammalian cell systems, promoters from mammalian genes or from mammalian viruses are generally preferred. If it is necessary to generate a cell line that contains multiple copies of the sequence encoding a polypeptide,  
20 vectors based on SV40 or EBV may be advantageously used with an appropriate selectable marker.

In bacterial systems, any of a number of expression vectors may be selected depending upon the use intended for the expressed polypeptide. For example, when large quantities are needed, for example for the induction of antibodies, vectors which direct high  
25 level expression of fusion proteins that are readily purified may be used. Such vectors include, but are not limited to, the multifunctional *E. coli* cloning and expression vectors such as BLUESCRIPT (Stratagene), in which the sequence encoding the polypeptide of interest may be ligated into the vector in frame with sequences for the amino-terminal Met and the subsequent 7 residues of .beta.-galactosidase so that a hybrid protein is produced;

pIN vectors (Van Heeke, G. and S. M. Schuster (1989) *J. Biol. Chem.* 264:5503-5509); and the like. pGEX Vectors (Promega, Madison, Wis.) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. Proteins made in such systems may be designed to include heparin, thrombin, or factor XA protease cleavage sites so that the cloned polypeptide of interest can be released from the GST moiety at will.

In the yeast, *Saccharomyces cerevisiae*, a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase, and PGH may be used. For reviews, see Ausubel et al. (supra) and Grant et al. (1987) *Methods Enzymol.* 153:516-544.

In cases where plant expression vectors are used, the expression of sequences encoding polypeptides may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV may be used alone or in combination with the omega leader sequence from TMV (Takamatsu, N. (1987) *EMBO J.* 6:307-311. Alternatively, plant promoters such as the small subunit of RUBISCO or heat shock promoters may be used (Coruzzi, G. et al. (1984) *EMBO J.* 3:1671-1680; Broglie, R. et al. (1984) *Science* 224:838-843; and Winter, J. et al. (1991) *Results Probl. Cell Differ.* 17:85-105). These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. Such techniques are described in a number of generally available reviews (see, for example, Hobbs, S. or Murry, L. E. in McGraw Hill Yearbook of Science and Technology (1992) McGraw Hill, New York, N.Y.; pp. 191-196).

An insect system may also be used to express a polypeptide of interest. For example, in one such system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes in *Spodoptera frugiperda* cells or in *Trichoplusia* larvae. The sequences encoding the polypeptide may be cloned into a non-essential region of the virus, such as the polyhedrin gene, and placed under control of the

polyhedrin promoter. Successful insertion of the polypeptide-encoding sequence will render the polyhedrin gene inactive and produce recombinant virus lacking coat protein. The recombinant viruses may then be used to infect, for example, *S. frugiperda* cells or *Trichoplusia* larvae in which the polypeptide of interest may be expressed (Engelhard, E. K. et al. (1994) *Proc. Natl. Acad. Sci.* 91 :3224-3227).

In mammalian host cells, a number of viral-based expression systems are generally available. For example, in cases where an adenovirus is used as an expression vector, sequences encoding a polypeptide of interest may be ligated into an adenovirus transcription/translation complex consisting of the late promoter and tripartite leader sequence. Insertion in a non-essential E1 or E3 region of the viral genome may be used to obtain a viable virus which is capable of expressing the polypeptide in infected host cells (Logan, J. and Shenk, T. (1984) *Proc. Natl. Acad. Sci.* 81:3655-3659). In addition, transcription enhancers, such as the Rous sarcoma virus (RSV) enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be used to achieve more efficient translation of sequences encoding a polypeptide of interest. Such signals include the ATG initiation codon and adjacent sequences. In cases where sequences encoding the polypeptide, its initiation codon, and upstream sequences are inserted into the appropriate expression vector, no additional transcriptional or translational control signals may be needed. However, in cases where only coding sequence, or a portion thereof, is inserted, exogenous translational control signals including the ATG initiation codon should be provided. Furthermore, the initiation codon should be in the correct reading frame to ensure translation of the entire insert. Exogenous translational elements and initiation codons may be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers which are appropriate for the particular cell system which is used, such as those described in the literature (Scharf, D. et al. (1994) *Results Probl. Cell Differ.* 20:125-162).

In addition, a host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired

fashion. Such modifications of the polypeptide include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation, and acylation. Post-translational processing which cleaves a "prepro" form of the protein may also be used to facilitate correct insertion, folding and/or function. Different host cells such as CHO, COS, HeLa, MDCK, HEK293, and WI38, which have specific cellular machinery and characteristic mechanisms for such post-translational activities, may be chosen to ensure the correct modification and processing of the foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is generally preferred. For example, cell lines which stably express a polynucleotide of interest may be transformed using expression vectors which may contain viral origins of replication and/or endogenous expression elements and a selectable marker gene on the same or on a separate vector. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clones of stably transformed cells may be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine kinase (Wigler, M. et al. (1977) *Cell* 11:223-32) and adenine phosphoribosyltransferase (Lowy, I. et al. (1990) *Cell* 22:817-23) genes which can be employed in tk.sup.- or aprt.sup.- cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, dhfr which confers resistance to methotrexate (Wigler, M. et al. (1980) *Proc. Natl. Acad. Sci.* 77:3567-70); npt, which confers resistance to the aminoglycosides, neomycin and G-418 (Colbere-Garapin, F. et al (1981) *J. Mol. Biol.* 150:1-14); and als or pat, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, *supra*). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan, or hisD, which allows cells to utilize histinol in place of histidine (Hartman, S. C. and R. C.

Mulligan (1988) *Proc. Natl. Acad. Sci.* 85:8047-51). The use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate GUS, and luciferase and its substrate luciferin, being widely used not only to identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C. A. et al. (1995) *Methods Mol. Biol.* 55:121-131).

Although the presence/absence of marker gene expression suggests that the gene of interest is also present, its presence and expression may need to be confirmed. For example, if the sequence encoding a polypeptide is inserted within a marker gene sequence, recombinant cells containing sequences can be identified by the absence of marker gene function. Alternatively, a marker gene can be placed in tandem with a polypeptide-encoding sequence under the control of a single promoter. Expression of the marker gene in response to induction or selection usually indicates expression of the tandem gene as well.

Alternatively, host cells that contain and express a desired polynucleotide sequence may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridizations and protein bioassay or immunoassay techniques which include, for example, membrane, solution, or chip based technologies for the detection and/or quantification of nucleic acid or protein.

A variety of protocols for detecting and measuring the expression of polynucleotide-encoded products, using either polyclonal or monoclonal antibodies specific for the product are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescence activated cell sorting (FACS). A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on a given polypeptide may be preferred for some applications, but a competitive binding assay may also be employed. These and other assays are described, among other places, in Hampton, R. et al. (1990; *Serological Methods, a Laboratory Manual*, APS Press, St Paul, Minn.) and Maddox, D. E. et al. (1983; *J. Exp. Med.* 158:1211-1216).

A wide variety of labels and conjugation techniques are known by those skilled in the art and may be used in various nucleic acid and amino acid assays. Means for producing labeled hybridization or PCR probes for detecting sequences related to polynucleotides include oligolabeling, nick translation, end-labeling or PCR amplification using a labeled nucleotide. Alternatively, the sequences, or any portions thereof may be cloned into a vector for the production of an mRNA probe. Such vectors are known in the art, are commercially available, and may be used to synthesize RNA probes in vitro by addition of an appropriate RNA polymerase such as T7, T3, or SP6 and labeled nucleotides. These procedures may be conducted using a variety of commercially available kits. Suitable reporter molecules or labels, which may be used include radionuclides, enzymes, fluorescent, chemiluminescent, or chromogenic agents as well as substrates, cofactors, inhibitors, magnetic particles, and the like.

Host cells transformed with a polynucleotide sequence of interest may be cultured under conditions suitable for the expression and recovery of the protein from cell culture. The protein produced by a recombinant cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing polynucleotides of the invention may be designed to contain signal sequences which direct secretion of the encoded polypeptide through a prokaryotic or eukaryotic cell membrane. Other recombinant constructions may be used to join sequences encoding a polypeptide of interest to nucleotide sequence encoding a polypeptide domain which will facilitate purification of soluble proteins. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp., Seattle, Wash.). The inclusion of cleavable linker sequences such as those specific for Factor XA or enterokinase (Invitrogen, San Diego, Calif.) between the purification domain and the encoded polypeptide may be used to facilitate purification. One such expression vector provides for expression of a fusion protein containing a polypeptide of interest and a

nucleic acid encoding 6 histidine residues preceding a thioredoxin or an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography) as described in Porath, J. et al. (1992, *Prot. Exp. Purif.* 3:263-281) while the enterokinase cleavage site provides a means for purifying the desired polypeptide from the fusion protein. A discussion of vectors which contain fusion proteins is provided in Kroll, D. J. et al. (1993; *DNA Cell Biol.* 12:441-453).

In addition to recombinant production methods, polypeptides of the invention, and fragments thereof, may be produced by direct peptide synthesis using solid-phase techniques (Merrifield J. (1963) *J. Am. Chem. Soc.* 85:2149-2154). Protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer). Alternatively, various fragments may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

#### Antibody Compositions, Fragments Thereof and Other Binding Agents

According to another aspect, the present invention further provides binding agents, such as antibodies and antigen-binding fragments thereof, that exhibit immunological binding to a tumor polypeptide disclosed herein, or to a portion, variant or derivative thereof. An antibody, or antigen-binding fragment thereof, is said to "specifically bind," "immunologically bind," and/or is "immunologically reactive" to a polypeptide of the invention if it reacts at a detectable level (within, for example, an ELISA assay) with the polypeptide, and does not react detectably with unrelated polypeptides under similar conditions.

Immunological binding, as used in this context, generally refers to the non-covalent interactions of the type which occur between an immunoglobulin molecule and an antigen for which the immunoglobulin is specific. The strength, or affinity of immunological binding interactions can be expressed in terms of the dissociation constant ( $K_d$ ) of the interaction, wherein a smaller  $K_d$  represents a greater affinity. Immunological binding properties of selected polypeptides can be quantified using methods well known in

the art. One such method entails measuring the rates of antigen-binding site/antigen complex formation and dissociation, wherein those rates depend on the concentrations of the complex partners, the affinity of the interaction, and on geometric parameters that equally influence the rate in both directions. Thus, both the "on rate constant" ( $K_{on}$ ) and the "off rate constant" ( $K_{off}$ ) can be determined by calculation of the concentrations and the actual rates of association and dissociation. The ratio of  $K_{off}/K_{on}$  enables cancellation of all parameters not related to affinity, and is thus equal to the dissociation constant  $K_d$ . See, generally, Davies et al. (1990) Annual Rev. Biochem. 59:439-473.

An "antigen-binding site," or "binding portion" of an antibody refers to the part of the immunoglobulin molecule that participates in antigen binding. The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs."

Binding agents may be further capable of differentiating between patients with and without a cancer, such as lung cancer, using the representative assays provided herein. For example, antibodies or other binding agents that bind to a tumor protein will preferably generate a signal indicating the presence of a cancer in at least about 20% of patients with the disease, more preferably at least about 30% of patients. Alternatively, or in addition, the antibody will generate a negative signal indicating the absence of the disease in at least about 90% of individuals without the cancer. To determine whether a



binding agent satisfies this requirement, biological samples (e.g., blood, sera, sputum, urine and/or tumor biopsies) from patients with and without a cancer (as determined using standard clinical tests) may be assayed as described herein for the presence of polypeptides that bind to the binding agent. Preferably, a statistically significant number of samples with and without the disease will be assayed. Each binding agent should satisfy the above criteria; however, those of ordinary skill in the art will recognize that binding agents may be used in combination to improve sensitivity.

Any agent that satisfies the above requirements may be a binding agent. For example, a binding agent may be a ribosome, with or without a peptide component, an RNA molecule or a polypeptide. In a preferred embodiment, a binding agent is an antibody or an antigen-binding fragment thereof. Antibodies may be prepared by any of a variety of techniques known to those of ordinary skill in the art. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, antibodies can be produced by cell culture techniques, including the generation of monoclonal antibodies as described herein, or via transfection of antibody genes into suitable bacterial or mammalian cell hosts, in order to allow for the production of recombinant antibodies. In one technique, an immunogen comprising the polypeptide is initially injected into any of a wide variety of mammals (e.g., mice, rats, rabbits, sheep or goats). In this step, the polypeptides of this invention may serve as the immunogen without modification. Alternatively, particularly for relatively short polypeptides, a superior immune response may be elicited if the polypeptide is joined to a carrier protein, such as bovine serum albumin or keyhole limpet hemocyanin. The immunogen is injected into the animal host, preferably according to a predetermined schedule incorporating one or more booster immunizations, and the animals are bled periodically. Polyclonal antibodies specific for the polypeptide may then be purified from such antisera by, for example, affinity chromatography using the polypeptide coupled to a suitable solid support.

Monoclonal antibodies specific for an antigenic polypeptide of interest may be prepared, for example, using the technique of Kohler and Milstein, *Eur. J. Immunol.* 6:511-519, 1976, and improvements thereto. Briefly, these methods involve the

- preparation of immortal cell lines capable of producing antibodies having the desired specificity (*i.e.*, reactivity with the polypeptide of interest). Such cell lines may be produced, for example, from spleen cells obtained from an animal immunized as described above. The spleen cells are then immortalized by, for example, fusion with a myeloma cell
- 5 fusion partner, preferably one that is syngeneic with the immunized animal. A variety of fusion techniques may be employed. For example, the spleen cells and myeloma cells may be combined with a nonionic detergent for a few minutes and then plated at low density on a selective medium that supports the growth of hybrid cells, but not myeloma cells. A preferred selection technique uses HAT (hypoxanthine, aminopterin, thymidine) selection.
- 10 After a sufficient time, usually about 1 to 2 weeks, colonies of hybrids are observed. Single colonies are selected and their culture supernatants tested for binding activity against the polypeptide. Hybridomas having high reactivity and specificity are preferred.

- Monoclonal antibodies may be isolated from the supernatants of growing hybridoma colonies. In addition, various techniques may be employed to enhance the yield,
- 15 such as injection of the hybridoma cell line into the peritoneal cavity of a suitable vertebrate host, such as a mouse. Monoclonal antibodies may then be harvested from the ascites fluid or the blood. Contaminants may be removed from the antibodies by conventional techniques, such as chromatography, gel filtration, precipitation, and extraction. The polypeptides of this invention may be used in the purification process in,
- 20 for example, an affinity chromatography step.

- A number of therapeutically useful molecules are known in the art which comprise antigen-binding sites that are capable of exhibiting immunological binding properties of an antibody molecule. The proteolytic enzyme papain preferentially cleaves IgG molecules to yield several fragments, two of which (the "F(ab)" fragments) each
- 25 comprise a covalent heterodimer that includes an intact antigen-binding site. The enzyme pepsin is able to cleave IgG molecules to provide several fragments, including the "F(ab')<sub>2</sub>" fragment which comprises both antigen-binding sites. An "Fv" fragment can be produced by preferential proteolytic cleavage of an IgM, and on rare occasions IgG or IgA immunoglobulin molecule. Fv fragments are, however, more commonly derived using

recombinant techniques known in the art. The Fv fragment includes a non-covalent  $V_H::V_L$  heterodimer including an antigen-binding site which retains much of the antigen recognition and binding capabilities of the native antibody molecule. Inbar et al. (1972) Proc. Nat. Acad. Sci. USA 69:2659-2662; Hochman et al. (1976) Biochem 15:2706-2710; and Ehrlich et al. (1980) Biochem 19:4091-4096.

5 A single chain Fv ("sFv") polypeptide is a covalently linked  $V_H::V_L$  heterodimer which is expressed from a gene fusion including  $V_H$ - and  $V_L$ -encoding genes linked by a peptide-encoding linker. Huston et al. (1988) Proc. Nat. Acad. Sci. USA 85(16):5879-5883. A number of methods have been described to discern chemical  
10 structures for converting the naturally aggregated--but chemically separated--light and heavy polypeptide chains from an antibody V region into an sFv molecule which will fold into a three dimensional structure substantially similar to the structure of an antigen-binding site. See, e.g., U.S. Pat. Nos. 5,091,513 and 5,132,405, to Huston et al.; and U.S. Pat. No. 4,946,778, to Ladner et al.

15 Each of the above-described molecules includes a heavy chain and a light chain CDR set, respectively interposed between a heavy chain and a light chain FR set which provide support to the CDRS and define the spatial relationship of the CDRs relative to each other. As used herein, the term "CDR set" refers to the three hypervariable regions of a heavy or light chain V region. Proceeding from the N-terminus of a heavy or light  
20 chain, these regions are denoted as "CDR1," "CDR2," and "CDR3" respectively. An antigen-binding site, therefore, includes six CDRs, comprising the CDR set from each of a heavy and a light chain V region. A polypeptide comprising a single CDR, (e.g., a CDR1, CDR2 or CDR3) is referred to herein as a "molecular recognition unit." Crystallographic  
25 analysis of a number of antigen-antibody complexes has demonstrated that the amino acid residues of CDRs form extensive contact with bound antigen, wherein the most extensive antigen contact is with the heavy chain CDR3. Thus, the molecular recognition units are primarily responsible for the specificity of an antigen-binding site.

As used herein, the term "FR set" refers to the four flanking amino acid sequences which frame the CDRs of a CDR set of a heavy or light chain V region. Some

FR residues may contact bound antigen; however, FRs are primarily responsible for folding the V region into the antigen-binding site, particularly the FR residues directly adjacent to the CDRs. Within FRs, certain amino residues and certain structural features are very highly conserved. In this regard, all V region sequences contain an internal disulfide loop of around 90 amino acid residues. When the V regions fold into a binding-site, the CDRs are displayed as projecting loop motifs which form an antigen-binding surface. It is generally recognized that there are conserved structural regions of FRs which influence the folded shape of the CDR loops into certain "canonical" structures--regardless of the precise CDR amino acid sequence. Further, certain FR residues are known to participate in non-covalent interdomain contacts which stabilize the interaction of the antibody heavy and light chains.

A number of "humanized" antibody molecules comprising an antigen-binding site derived from a non-human immunoglobulin have been described, including chimeric antibodies having rodent V regions and their associated CDRs fused to human constant domains (Winter et al. (1991) Nature 349:293-299; Lobuglio et al. (1989) Proc. Nat. Acad. Sci. USA 86:4220-4224; Shaw et al. (1987) J Immunol. 138:4534-4538; and Brown et al. (1987) Cancer Res. 47:3577-3583), rodent CDRs grafted into a human supporting FR prior to fusion with an appropriate human antibody constant domain (Riechmann et al. (1988) Nature 332:323-327; Verhoeyen et al. (1988) Science 239:1534-1536; and Jones et al. (1986) Nature 321:522-525), and rodent CDRs supported by recombinantly veneered rodent FRs (European Patent Publication No. 519,596, published Dec. 23, 1992). These "humanized" molecules are designed to minimize unwanted immunological response toward rodent antihuman antibody molecules which limits the duration and effectiveness of therapeutic applications of those moieties in human recipients.

As used herein, the terms "veneered FRs" and "recombinantly veneered FRs" refer to the selective replacement of FR residues from, *e.g.*, a rodent heavy or light chain V region, with human FR residues in order to provide a xenogeneic molecule comprising an antigen-binding site which retains substantially all of the native FR polypeptide folding structure. Veneering techniques are based on the understanding that the

ligand binding characteristics of an antigen-binding site are determined primarily by the structure and relative disposition of the heavy and light chain CDR sets within the antigen-binding surface. Davies et al. (1990) *Ann. Rev. Biochem.* 59:439-473. Thus, antigen binding specificity can be preserved in a humanized antibody only wherein the CDR structures, their interaction with each other, and their interaction with the rest of the V region domains are carefully maintained. By using veneering techniques, exterior (*e.g.*, solvent-accessible) FR residues which are readily encountered by the immune system are selectively replaced with human residues to provide a hybrid molecule that comprises either a weakly immunogenic, or substantially non-immunogenic veneered surface.

The process of veneering makes use of the available sequence data for human antibody variable domains compiled by Kabat et al., in *Sequences of Proteins of Immunological Interest*, 4th ed., (U.S. Dept. of Health and Human Services, U.S. Government Printing Office, 1987), updates to the Kabat database, and other accessible U.S. and foreign databases (both nucleic acid and protein). Solvent accessibilities of V region amino acids can be deduced from the known three-dimensional structure for human and murine antibody fragments. There are two general steps in veneering a murine antigen-binding site. Initially, the FRs of the variable domains of an antibody molecule of interest are compared with corresponding FR sequences of human variable domains obtained from the above-identified sources. The most homologous human V regions are then compared residue by residue to corresponding murine amino acids. The residues in the murine FR which differ from the human counterpart are replaced by the residues present in the human moiety using recombinant techniques well known in the art. Residue switching is only carried out with moieties which are at least partially exposed (solvent accessible), and care is exercised in the replacement of amino acid residues which may have a significant effect on the tertiary structure of V region domains, such as proline, glycine and charged amino acids.

In this manner, the resultant "veneered" murine antigen-binding sites are thus designed to retain the murine CDR residues, the residues substantially adjacent to the CDRs, the residues identified as buried or mostly buried (solvent inaccessible), the residues

believed to participate in non-covalent (*e.g.*, electrostatic and hydrophobic) contacts between heavy and light chain domains, and the residues from conserved structural regions of the FRs which are believed to influence the "canonical" tertiary structures of the CDR loops. These design criteria are then used to prepare recombinant nucleotide sequences which combine the CDRs of both the heavy and light chain of a murine antigen-binding site into human-appearing FRs that can be used to transfect mammalian cells for the expression of recombinant human antibodies which exhibit the antigen specificity of the murine antibody molecule.

In another embodiment of the invention, monoclonal antibodies of the present invention may be coupled to one or more therapeutic agents. Suitable agents in this regard include radionuclides, differentiation inducers, drugs, toxins, and derivatives thereof. Preferred radionuclides include  $^{90}\text{Y}$ ,  $^{123}\text{I}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{211}\text{At}$ , and  $^{212}\text{Bi}$ . Preferred drugs include methotrexate, and pyrimidine and purine analogs. Preferred differentiation inducers include phorbol esters and butyric acid. Preferred toxins include ricin, abrin, diphtheria toxin, cholera toxin, gelonin, *Pseudomonas* exotoxin, *Shigella* toxin, and pokeweed antiviral protein.

A therapeutic agent may be coupled (*e.g.*, covalently bonded) to a suitable monoclonal antibody either directly or indirectly (*e.g.*, via a linker group). A direct reaction between an agent and an antibody is possible when each possesses a substituent capable of reacting with the other. For example, a nucleophilic group, such as an amino or sulfhydryl group, on one may be capable of reacting with a carbonyl-containing group, such as an anhydride or an acid halide, or with an alkyl group containing a good leaving group (*e.g.*, a halide) on the other.

Alternatively, it may be desirable to couple a therapeutic agent and an antibody via a linker group. A linker group can function as a spacer to distance an antibody from an agent in order to avoid interference with binding capabilities. A linker group can also serve to increase the chemical reactivity of a substituent on an agent or an antibody, and thus increase the coupling efficiency. An increase in chemical reactivity may also

facilitate the use of agents, or functional groups on agents, which otherwise would not be possible.

It will be evident to those skilled in the art that a variety of bifunctional or polyfunctional reagents, both homo- and hetero-functional (such as those described in the catalog of the Pierce Chemical Co., Rockford, IL), may be employed as the linker group. Coupling may be effected, for example, through amino groups, carboxyl groups, sulfhydryl groups or oxidized carbohydrate residues. There are numerous references describing such methodology, *e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.

Where a therapeutic agent is more potent when free from the antibody portion of the immunoconjugates of the present invention, it may be desirable to use a linker group which is cleavable during or upon internalization into a cell. A number of different cleavable linker groups have been described. The mechanisms for the intracellular release of an agent from these linker groups include cleavage by reduction of a disulfide bond (*e.g.*, U.S. Patent No. 4,489,710, to Spitler), by irradiation of a photolabile bond (*e.g.*, U.S. Patent No. 4,625,014, to Senter et al.), by hydrolysis of derivatized amino acid side chains (*e.g.*, U.S. Patent No. 4,638,045, to Kohn et al.), by serum complement-mediated hydrolysis (*e.g.*, U.S. Patent No. 4,671,958, to Rodwell et al.), and acid-catalyzed hydrolysis (*e.g.*, U.S. Patent No. 4,569,789, to Blattler et al.).

It may be desirable to couple more than one agent to an antibody. In one embodiment, multiple molecules of an agent are coupled to one antibody molecule. In another embodiment, more than one type of agent may be coupled to one antibody. Regardless of the particular embodiment, immunoconjugates with more than one agent may be prepared in a variety of ways. For example, more than one agent may be coupled directly to an antibody molecule, or linkers that provide multiple sites for attachment can be used. Alternatively, a carrier can be used.

A carrier may bear the agents in a variety of ways, including covalent bonding either directly or via a linker group. Suitable carriers include proteins such as albumins (*e.g.*, U.S. Patent No. 4,507,234, to Kato et al.), peptides and polysaccharides such as aminodextran (*e.g.*, U.S. Patent No. 4,699,784, to Shih et al.). A carrier may also

bear an agent by noncovalent bonding or by encapsulation, such as within a liposome vesicle (e.g., U.S. Patent Nos. 4,429,008 and 4,873,088). Carriers specific for radionuclide agents include radiohalogenated small molecules and chelating compounds. For example, U.S. Patent No. 4,735,792 discloses representative radiohalogenated small molecules and their synthesis. A radionuclide chelate may be formed from chelating compounds that include those containing nitrogen and sulfur atoms as the donor atoms for binding the metal, or metal oxide, radionuclide. For example, U.S. Patent No. 4,673,562, to Davison et al. discloses representative chelating compounds and their synthesis.

### T Cell Compositions

10 The present invention, in another aspect, provides T cells specific for a tumor polypeptide disclosed herein, or for a variant or derivative thereof. Such cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood, or a fraction of bone marrow or peripheral blood of a patient, using a commercially available cell separation system, such as 15 the Isolex™ System, available from Nexell Therapeutics, Inc. (Irvine, CA; see also U.S. Patent No. 5,240,856; U.S. Patent No. 5,215,926; WO 89/06280; WO 91/16116 and WO 92/07243). Alternatively, T cells may be derived from related or unrelated humans, non-human mammals, cell lines or cultures.

T cells may be stimulated with a polypeptide, polynucleotide encoding a polypeptide and/or an antigen presenting cell (APC) that expresses such a polypeptide. 20 Such stimulation is performed under conditions and for a time sufficient to permit the generation of T cells that are specific for the polypeptide of interest. Preferably, a tumor polypeptide or polynucleotide of the invention is present within a delivery vehicle, such as a microsphere, to facilitate the generation of specific T cells.

25 T cells are considered to be specific for a polypeptide of the present invention if the T cells specifically proliferate, secrete cytokines or kill target cells coated with the polypeptide or expressing a gene encoding the polypeptide. T cell specificity may be evaluated using any of a variety of standard techniques. For example, within a



chromium release assay or proliferation assay, a stimulation index of more than two fold increase in lysis and/or proliferation, compared to negative controls, indicates T cell specificity. Such assays may be performed, for example, as described in Chen et al., *Cancer Res.* 54:1065-1070, 1994. Alternatively, detection of the proliferation of T cells may be accomplished by a variety of known techniques. For example, T cell proliferation can be detected by measuring an increased rate of DNA synthesis (*e.g.*, by pulse-labeling cultures of T cells with tritiated thymidine and measuring the amount of tritiated thymidine incorporated into DNA). Contact with a tumor polypeptide (100 ng/ml - 100 µg/ml, preferably 200 ng/ml - 25 µg/ml) for 3 - 7 days will typically result in at least a two fold increase in proliferation of the T cells. Contact as described above for 2-3 hours should result in activation of the T cells, as measured using standard cytokine assays in which a two fold increase in the level of cytokine release (*e.g.*, TNF or IFN-γ) is indicative of T cell activation (*see* Coligan et al., *Current Protocols in Immunology*, vol. 1, Wiley Interscience (Greene 1998)). T cells that have been activated in response to a tumor polypeptide, polynucleotide or polypeptide-expressing APC may be CD4<sup>+</sup> and/or CD8<sup>+</sup>. Tumor polypeptide-specific T cells may be expanded using standard techniques. Within preferred embodiments, the T cells are derived from a patient, a related donor or an unrelated donor, and are administered to the patient following stimulation and expansion.

For therapeutic purposes, CD4<sup>+</sup> or CD8<sup>+</sup> T cells that proliferate in response to a tumor polypeptide, polynucleotide or APC can be expanded in number either *in vitro* or *in vivo*. Proliferation of such T cells *in vitro* may be accomplished in a variety of ways. For example, the T cells can be re-exposed to a tumor polypeptide, or a short peptide corresponding to an immunogenic portion of such a polypeptide, with or without the addition of T cell growth factors, such as interleukin-2, and/or stimulator cells that synthesize a tumor polypeptide. Alternatively, one or more T cells that proliferate in the presence of the tumor polypeptide can be expanded in number by cloning. Methods for cloning cells are well known in the art, and include limiting dilution.

### Pharmaceutical Compositions

In additional embodiments, the present invention concerns formulation of one or more of the polynucleotide, polypeptide, T-cell and/or antibody compositions disclosed herein in pharmaceutically-acceptable carriers for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy.

It will be understood that, if desired, a composition as disclosed herein may be administered in combination with other agents as well, such as, *e.g.*, other proteins or polypeptides or various pharmaceutically-active agents. In fact, there is virtually no limit to other components that may also be included, given that the additional agents do not cause a significant adverse effect upon contact with the target cells or host tissues. The compositions may thus be delivered along with various other agents as required in the particular instance. Such compositions may be purified from host cells or other biological sources, or alternatively may be chemically synthesized as described herein. Likewise, such compositions may further comprise substituted or derivatized RNA or DNA compositions.

Therefore, in another aspect of the present invention, pharmaceutical compositions are provided comprising one or more of the polynucleotide, polypeptide, antibody, and/or T-cell compositions described herein in combination with a physiologically acceptable carrier. In certain preferred embodiments, the pharmaceutical compositions of the invention comprise immunogenic polynucleotide and/or polypeptide compositions of the invention for use in prophylactic and therapeutic vaccine applications. Vaccine preparation is generally described in, for example, M.F. Powell and M.J. Newman, eds., "Vaccine Design (the subunit and adjuvant approach)," Plenum Press (NY, 1995). Generally, such compositions will comprise one or more polynucleotide and/or polypeptide compositions of the present invention in combination with one or more immunostimulants.

It will be apparent that any of the pharmaceutical compositions described herein can contain pharmaceutically acceptable salts of the polynucleotides and polypeptides of the invention. Such salts can be prepared, for example, from pharmaceutically acceptable non-toxic bases, including organic bases (*e.g.*, salts of primary,

secondary and tertiary amines and basic amino acids) and inorganic bases (e.g., sodium, potassium, lithium, ammonium, calcium and magnesium salts).

In another embodiment, illustrative immunogenic compositions, e.g., vaccine compositions, of the present invention comprise DNA encoding one or more of the polypeptides as described above, such that the polypeptide is generated *in situ*. As noted above, the polynucleotide may be administered within any of a variety of delivery systems known to those of ordinary skill in the art. Indeed, numerous gene delivery techniques are well known in the art, such as those described by Rolland, *Crit. Rev. Therap. Drug Carrier Systems* 15:143-198, 1998, and references cited therein. Appropriate polynucleotide expression systems will, of course, contain the necessary regulatory DNA regulatory sequences for expression in a patient (such as a suitable promoter and terminating signal). Alternatively, bacterial delivery systems may involve the administration of a bacterium (such as *Bacillus-Calmette-Guerrin*) that expresses an immunogenic portion of the polypeptide on its cell surface or secretes such an epitope.

Therefore, in certain embodiments, polynucleotides encoding immunogenic polypeptides described herein are introduced into suitable mammalian host cells for expression using any of a number of known viral-based systems. In one illustrative embodiment, retroviruses provide a convenient and effective platform for gene delivery systems. A selected nucleotide sequence encoding a polypeptide of the present invention can be inserted into a vector and packaged in retroviral particles using techniques known in the art. The recombinant virus can then be isolated and delivered to a subject. A number of illustrative retroviral systems have been described (e.g., U.S. Pat. No. 5,219,740; Miller and Rosman (1989) *BioTechniques* 7:980-990; Miller, A. D. (1990) *Human Gene Therapy* 1:5-14; Scarpa et al. (1991) *Virology* 180:849-852; Burns et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:8033-8037; and Boris-Lawrie and Temin (1993) *Cur. Opin. Genet. Develop.* 3:102-109.

In addition, a number of illustrative adenovirus-based systems have also been described. Unlike retroviruses which integrate into the host genome, adenoviruses persist extrachromosomally thus minimizing the risks associated with insertional

mutagenesis (Haj-Ahmad and Graham (1986) *J. Virol.* 57:267-274; Bett et al. (1993) *J. Virol.* 67:5911-5921; Mittereder et al. (1994) *Human Gene Therapy* 5:717-729; Seth et al. (1994) *J. Virol.* 68:933-940; Barr et al. (1994) *Gene Therapy* 1:51-58; Berkner, K. L. (1988) *BioTechniques* 6:616-629; and Rich et al. (1993) *Human Gene Therapy* 4:461-476).

5 Various adeno-associated virus (AAV) vector systems have also been developed for polynucleotide delivery. AAV vectors can be readily constructed using techniques well known in the art. See, e.g., U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 92/01070 and WO 93/03769; Lebkowski et al. (1988) *Molec. Cell. Biol.* 8:3988-3996; Vincent et al. (1990) *Vaccines* 90 (Cold Spring Harbor Laboratory Press); Carter, B. J. (1992) *Current Opinion in Biotechnology* 3:533-539; 10 Muzyczka, N. (1992) *Current Topics in Microbiol. and Immunol.* 158:97-129; Kotin, R. M. (1994) *Human Gene Therapy* 5:793-801; Shelling and Smith (1994) *Gene Therapy* 1:165-169; and Zhou et al. (1994) *J. Exp. Med.* 179:1867-1875.

Additional viral vectors useful for delivering the polynucleotides encoding 15 polypeptides of the present invention by gene transfer include those derived from the pox family of viruses, such as vaccinia virus and avian poxvirus. By way of example, vaccinia virus recombinants expressing the novel molecules can be constructed as follows. The DNA encoding a polypeptide is first inserted into an appropriate vector so that it is adjacent to a vaccinia promoter and flanking vaccinia DNA sequences, such as the sequence 20 encoding thymidine kinase (TK). This vector is then used to transfect cells which are simultaneously infected with vaccinia. Homologous recombination serves to insert the vaccinia promoter plus the gene encoding the polypeptide of interest into the viral genome. The resulting TK.sup.(-) recombinant can be selected by culturing the cells in the presence of 5-bromodeoxyuridine and picking viral plaques resistant thereto.

25 A vaccinia-based infection/transfection system can be conveniently used to provide for inducible, transient expression or coexpression of one or more polypeptides described herein in host cells of an organism. In this particular system, cells are first infected in vitro with a vaccinia virus recombinant that encodes the bacteriophage T7 RNA polymerase. This polymerase displays exquisite specificity in that it only transcribes

templates bearing T7 promoters. Following infection, cells are transfected with the polynucleotide or polynucleotides of interest, driven by a T7 promoter. The polymerase expressed in the cytoplasm from the vaccinia virus recombinant transcribes the transfected DNA into RNA which is then translated into polypeptide by the host translational machinery. The method provides for high level, transient, cytoplasmic production of large quantities of RNA and its translation products. See, *e.g.*, Elroy-Stein and Moss, *Proc. Natl. Acad. Sci. USA* (1990) 87:6743-6747; Fuerst et al. *Proc. Natl. Acad. Sci. USA* (1986) 83:8122-8126.

Alternatively, avipoxviruses, such as the fowlpox and canarypox viruses, can also be used to deliver the coding sequences of interest. Recombinant avipox viruses, expressing immunogens from mammalian pathogens, are known to confer protective immunity when administered to non-avian species. The use of an Avipox vector is particularly desirable in human and other mammalian species since members of the Avipox genus can only productively replicate in susceptible avian species and therefore are not infective in mammalian cells. Methods for producing recombinant Avipoxviruses are known in the art and employ genetic recombination, as described above with respect to the production of vaccinia viruses. See, *e.g.*, WO 91/12882; WO 89/03429; and WO 92/03545.

Any of a number of alphavirus vectors can also be used for delivery of polynucleotide compositions of the present invention, such as those vectors described in U.S. Patent Nos. 5,843,723; 6,015,686; 6,008,035 and 6,015,694. Certain vectors based on Venezuelan Equine Encephalitis (VEE) can also be used, illustrative examples of which can be found in U.S. Patent Nos. 5,505,947 and 5,643,576.

Moreover, molecular conjugate vectors, such as the adenovirus chimeric vectors described in Michael et al. *J. Biol. Chem.* (1993) 268:6866-6869 and Wagner et al. *Proc. Natl. Acad. Sci. USA* (1992) 89:6099-6103, can also be used for gene delivery under the invention.

Additional illustrative information on these and other known viral-based delivery systems can be found, for example, in Fisher-Hoch et al., *Proc. Natl. Acad. Sci. USA* 86:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et

al., *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973; U.S. Patent No. 4,777,127; GB 2,200,651; EP 0,345,242; WO 91/02805; Berkner, *Biotechniques* 6:616-627, 1988; Rosenfeld et al., *Science* 252:431-434, 1991; Kolls et al., *Proc. Natl. Acad. Sci. USA* 91:215-219, 1994; Kass-Eisler et al., *Proc. Natl. Acad. Sci. USA* 90:11498-11502, 1993; Guzman et al., *Circulation* 88:2838-2848, 1993; and Guzman et al., *Cir. Res.* 73:1202-1207, 1993.

In certain embodiments, a polynucleotide may be integrated into the genome of a target cell. This integration may be in the specific location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the polynucleotide may be stably maintained in the cell as a separate, episomal segment of DNA. Such polynucleotide segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. The manner in which the expression construct is delivered to a cell and where in the cell the polynucleotide remains is dependent on the type of expression construct employed.

In another embodiment of the invention, a polynucleotide is administered/delivered as "naked" DNA, for example as described in Ulmer et al., *Science* 259:1745-1749, 1993 and reviewed by Cohen, *Science* 259:1691-1692, 1993. The uptake of naked DNA may be increased by coating the DNA onto biodegradable beads, which are efficiently transported into the cells.

In still another embodiment, a composition of the present invention can be delivered via a particle bombardment approach, many of which have been described. In one illustrative example, gas-driven particle acceleration can be achieved with devices such as those manufactured by Powderject Pharmaceuticals PLC (Oxford, UK) and Powderject Vaccines Inc. (Madison, WI), some examples of which are described in U.S. Patent Nos. 5,846,796; 6,010,478; 5,865,796; 5,584,807; and EP Patent No. 0500 799. This approach offers a needle-free delivery approach wherein a dry powder formulation of microscopic particles, such as polynucleotide or polypeptide particles, are accelerated to high speed

within a helium gas jet generated by a hand held device, propelling the particles into a target tissue of interest.

In a related embodiment, other devices and methods that may be useful for gas-driven needle-less injection of compositions of the present invention include those provided by Bioject, Inc. (Portland, OR), some examples of which are described in U.S. Patent Nos. 4,790,824; 5,064,413; 5,312,335; 5,383,851; 5,399,163; 5,520,639 and 5,993,412.

According to another embodiment, the pharmaceutical compositions described herein will comprise one or more immunostimulants in addition to the immunogenic polynucleotide, polypeptide, antibody, T-cell and/or APC compositions of this invention. An immunostimulant refers to essentially any substance that enhances or potentiates an immune response (antibody and/or cell-mediated) to an exogenous antigen. One preferred type of immunostimulant comprises an adjuvant. Many adjuvants contain a substance designed to protect the antigen from rapid catabolism, such as aluminum hydroxide or mineral oil, and a stimulator of immune responses, such as lipid A, *Bordetella pertussis* or *Mycobacterium tuberculosis* derived proteins. Certain adjuvants are commercially available as, for example, Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, MI); Merck Adjuvant 65 (Merck and Company, Inc., Rahway, NJ); AS-2 (SmithKline Beecham, Philadelphia, PA); aluminum salts such as aluminum hydroxide gel (alum) or aluminum phosphate; salts of calcium, iron or zinc; an insoluble suspension of acylated tyrosine; acylated sugars; cationically or anionically derivatized polysaccharides; polyphosphazenes; biodegradable microspheres; monophosphoryl lipid A and quil A. Cytokines, such as GM-CSF, interleukin-2, -7, -12, and other like growth factors, may also be used as adjuvants.

Within certain embodiments of the invention, the adjuvant composition is preferably one that induces an immune response predominantly of the Th1 type. High levels of Th1-type cytokines (*e.g.*, IFN- $\gamma$ , TNF $\alpha$ , IL-2 and IL-12) tend to favor the induction of cell mediated immune responses to an administered antigen. In contrast, high levels of Th2-type cytokines (*e.g.*, IL-4, IL-5, IL-6 and IL-10) tend to favor the induction of

humoral immune responses. Following application of a vaccine as provided herein, a patient will support an immune response that includes Th1- and Th2-type responses. Within a preferred embodiment, in which a response is predominantly Th1-type, the level of Th1-type cytokines will increase to a greater extent than the level of Th2-type cytokines.

- 5 The levels of these cytokines may be readily assessed using standard assays. For a review of the families of cytokines, see Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173, 1989.

Certain preferred adjuvants for eliciting a predominantly Th1-type response include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A, together with an aluminum salt. MPL<sup>®</sup> adjuvants are available from Corixa Corporation (Seattle, WA; *see*, for example, US Patent Nos. 4,436,727; 4,877,611; 4,866,034 and 4,912,094). CpG-containing oligonucleotides (in which the CpG dinucleotide is unmethylated) also induce a predominantly Th1 response. Such oligonucleotides are well known and are described, for example, in WO 96/02555, WO 99/33488 and U.S. Patent Nos. 6,008,200 and 5,856,462. Immunostimulatory DNA sequences are also described, for example, by Sato et al., *Science* 273:352, 1996. Another preferred adjuvant comprises a saponin, such as Quil A, or derivatives thereof, including QS21 and QS7 (Aquila Biopharmaceuticals Inc., Framingham, MA); Escin; Digitonin; or *Gypsophila* or *Chenopodium quinoa* saponins. Other preferred formulations include more than one saponin in the adjuvant combinations of the present invention, for example combinations of at least two of the following group comprising QS21, QS7, Quil A,  $\beta$ -escin, or digitonin.

Alternatively the saponin formulations may be combined with vaccine vehicles composed of chitosan or other polycationic polymers, polylactide and polylactide-co-glycolide particles, poly-N-acetyl glucosamine-based polymer matrix, particles composed of polysaccharides or chemically modified polysaccharides, liposomes and lipid-based particles, particles composed of glycerol monoesters, etc. The saponins may also be formulated in the presence of cholesterol to form particulate structures such as liposomes or ISCOMs. Furthermore, the saponins may be formulated together with a polyoxyethylene



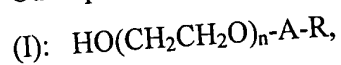
ether or ester, in either a non-particulate solution or suspension, or in a particulate structure such as a paucilamellar liposome or ISCOM. The saponins may also be formulated with excipients such as Carbopol<sup>R</sup> to increase viscosity, or may be formulated in a dry powder form with a powder excipient such as lactose.

5 In one preferred embodiment, the adjuvant system includes the combination of a monophosphoryl lipid A and a saponin derivative, such as the combination of QS21 and 3D-MPL<sup>®</sup> adjuvant, as described in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol, as described in WO 96/33739. Other preferred formulations comprise an oil-in-water emulsion and tocopherol. Another particularly preferred adjuvant formulation employing QS21, 3D-MPL<sup>®</sup> adjuvant and  
10 tocopherol in an oil-in-water emulsion is described in WO 95/17210.

Another enhanced adjuvant system involves the combination of a CpG-containing oligonucleotide and a saponin derivative particularly the combination of CpG and QS21 is disclosed in WO 00/09159. Preferably the formulation additionally comprises  
15 an oil in water emulsion and tocopherol.

Additional illustrative adjuvants for use in the pharmaceutical compositions of the invention include Montanide ISA 720 (Seppic, France), SAF (Chiron, California, United States), ISCOMS (CSL), MF-59 (Chiron), the SBAS series of adjuvants (*e.g.*, SBAS-2 or SBAS-4, available from SmithKline Beecham, Rixensart, Belgium), Detox  
20 (Enhanzyn<sup>®</sup>) (Corixa, Hamilton, MT), RC-529 (Corixa, Hamilton, MT) and other aminoalkyl glucosaminide 4-phosphates (AGPs), such as those described in pending U.S. Patent Application Serial Nos. 08/853,826 and 09/074,720, the disclosures of which are incorporated herein by reference in their entireties, and polyoxyethylene ether adjuvants such as those described in WO 99/52549A1.

25 Other preferred adjuvants include adjuvant molecules of the general formula



wherein,  $n$  is 1-50, A is a bond or  $-\text{C}(\text{O})-$ , R is  $\text{C}_{1-50}$  alkyl or Phenyl  $\text{C}_{1-50}$  alkyl.

One embodiment of the present invention consists of a vaccine formulation comprising a polyoxyethylene ether of general formula (I), wherein  $n$  is between 1 and 50,

preferably 4-24, most preferably 9; the *R* component is C<sub>1-50</sub>, preferably C<sub>4</sub>-C<sub>20</sub> alkyl and most preferably C<sub>12</sub> alkyl, and *A* is a bond. The concentration of the polyoxyethylene ethers should be in the range 0.1-20%, preferably from 0.1-10%, and most preferably in the range 0.1-1%. Preferred polyoxyethylene ethers are selected from the following group:

5 polyoxyethylene-9-lauryl ether, polyoxyethylene-9-stearyl ether, polyoxyethylene-8-stearyl ether, polyoxyethylene-4-lauryl ether, polyoxyethylene-35-lauryl ether, and polyoxyethylene-23-lauryl ether. Polyoxyethylene ethers such as polyoxyethylene lauryl ether are described in the Merck index (12<sup>th</sup> edition: entry 7717). These adjuvant molecules are described in WO 99/52549.

10 The polyoxyethylene ether according to the general formula (I) above may, if desired, be combined with another adjuvant. For example, a preferred adjuvant combination is preferably with CpG as described in the pending UK patent application GB 9820956.2.

According to another embodiment of this invention, an immunogenic

15 composition described herein is delivered to a host via antigen presenting cells (APCs), such as dendritic cells, macrophages, B cells, monocytes and other cells that may be engineered to be efficient APCs. Such cells may, but need not, be genetically modified to increase the capacity for presenting the antigen, to improve activation and/or maintenance of the T cell response, to have anti-tumor effects *per se* and/or to be immunologically

20 compatible with the receiver (*i.e.*, matched HLA haplotype). APCs may generally be isolated from any of a variety of biological fluids and organs, including tumor and peritumoral tissues, and may be autologous, allogeneic, syngeneic or xenogeneic cells.

Certain preferred embodiments of the present invention use dendritic cells or progenitors thereof as antigen-presenting cells. Dendritic cells are highly potent APCs

25 (Banchereau and Steinman, *Nature* 392:245-251, 1998) and have been shown to be effective as a physiological adjuvant for eliciting prophylactic or therapeutic antitumor immunity (*see* Timmerman and Levy, *Ann. Rev. Med.* 50:507-529, 1999). In general, dendritic cells may be identified based on their typical shape (stellate *in situ*, with marked cytoplasmic processes (dendrites) visible *in vitro*), their ability to take up, process and

present antigens with high efficiency and their ability to activate naïve T cell responses. Dendritic cells may, of course, be engineered to express specific cell-surface receptors or ligands that are not commonly found on dendritic cells *in vivo* or *ex vivo*, and such modified dendritic cells are contemplated by the present invention. As an alternative to  
5 dendritic cells, secreted vesicles antigen-loaded dendritic cells (called exosomes) may be used within a vaccine (*see* Zitvogel et al., *Nature Med.* 4:594-600, 1998).

Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells  
10 may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNF $\alpha$  to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNF $\alpha$ , CD40 ligand, LPS, flt3 ligand and/or other  
15 compound(s) that induce differentiation, maturation and proliferation of dendritic cells.

Dendritic cells are conveniently categorized as "immature" and "mature" cells, which allows a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as APC  
20 with a high capacity for antigen uptake and processing, which correlates with the high expression of Fc $\gamma$  receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (*e.g.*, CD54 and CD11) and costimulatory molecules (*e.g.*, CD40, CD80, CD86  
25 and 4-1BB).

APCs may generally be transfected with a polynucleotide of the invention (or portion or other variant thereof) such that the encoded polypeptide, or an immunogenic portion thereof, is expressed on the cell surface. Such transfection may take place *ex vivo*, and a pharmaceutical composition comprising such transfected cells may then be used for

therapeutic purposes, as described herein. Alternatively, a gene delivery vehicle that targets a dendritic or other antigen presenting cell may be administered to a patient, resulting in transfection that occurs *in vivo*. *In vivo* and *ex vivo* transfection of dendritic cells, for example, may generally be performed using any methods known in the art, such as those described in WO 97/24447, or the gene gun approach described by Mahvi et al.,  
 5 *Immunology and cell Biology* 75:456-460, 1997. Antigen loading of dendritic cells may be achieved by incubating dendritic cells or progenitor cells with the tumor polypeptide, DNA (naked or within a plasmid vector) or RNA; or with antigen-expressing recombinant bacterium or viruses (e.g., vaccinia, fowlpox, adenovirus or lentivirus vectors). Prior to  
 10 loading, the polypeptide may be covalently conjugated to an immunological partner that provides T cell help (e.g., a carrier molecule). Alternatively, a dendritic cell may be pulsed with a non-conjugated immunological partner, separately or in the presence of the polypeptide.

While any suitable carrier known to those of ordinary skill in the art may be employed in the pharmaceutical compositions of this invention, the type of carrier will  
 15 typically vary depending on the mode of administration. Compositions of the present invention may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, mucosal, intravenous, intracranial, intraperitoneal, subcutaneous and intramuscular administration.

Carriers for use within such pharmaceutical compositions are biocompatible,  
 20 and may also be biodegradable. In certain embodiments, the formulation preferably provides a relatively constant level of active component release. In other embodiments, however, a more rapid rate of release immediately upon administration may be desired. The formulation of such compositions is well within the level of ordinary skill in the art using known techniques. Illustrative carriers useful in this regard include microparticles of  
 25 poly(lactide-co-glycolide), polyacrylate, latex, starch, cellulose, dextran and the like. Other illustrative delayed-release carriers include supramolecular biovectors, which comprise a non-liquid hydrophilic core (e.g., a cross-linked polysaccharide or oligosaccharide) and, optionally, an external layer comprising an amphiphilic compound,

such as a phospholipid (*see e.g.*, U.S. Patent No. 5,151,254 and PCT applications WO 94/20078, WO/94/23701 and WO 96/06638). The amount of active compound contained within a sustained release formulation depends upon the site of implantation, the rate and expected duration of release and the nature of the condition to be treated or prevented.

5 In another illustrative embodiment, biodegradable microspheres (*e.g.*, polylactate polyglycolate) are employed as carriers for the compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344, 10 5,407,609 and 5,942,252. Modified hepatitis B core protein carrier systems, such as described in WO/99 40934, and references cited therein, will also be useful for many applications. Another illustrative carrier/delivery system employs a carrier comprising particulate-protein complexes, such as those described in U.S. Patent No. 5,928,647, which are capable of inducing a class I-restricted cytotoxic T lymphocyte responses in a host.

The pharmaceutical compositions of the invention will often further 15 comprise one or more buffers (*e.g.*, neutral buffered saline or phosphate buffered saline), carbohydrates (*e.g.*, glucose, mannose, sucrose or dextrans), mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide), solutes that render the formulation isotonic, hypotonic or weakly hypertonic with the blood of a recipient, 20 suspending agents, thickening agents and/or preservatives. Alternatively, compositions of the present invention may be formulated as a lyophilizate.

The pharmaceutical compositions described herein may be presented in unit-dose or multi-dose containers, such as sealed ampoules or vials. Such containers are typically sealed in such a way to preserve the sterility and stability of the formulation until 25 use. In general, formulations may be stored as suspensions, solutions or emulsions in oily or aqueous vehicles. Alternatively, a pharmaceutical composition may be stored in a freeze-dried condition requiring only the addition of a sterile liquid carrier immediately prior to use.

The development of suitable dosing and treatment regimens for using the particular compositions described herein in a variety of treatment regimens, including *e.g.*, oral, parenteral, intravenous, intranasal, and intramuscular administration and formulation, is well known in the art, some of which are briefly discussed below for general purposes of illustration.

In certain applications, the pharmaceutical compositions disclosed herein may be delivered *via* oral administration to an animal. As such, these compositions may be formulated with an inert diluent or with an assimilable edible carrier, or they may be enclosed in hard- or soft-shell gelatin capsule, or they may be compressed into tablets, or they may be incorporated directly with the food of the diet.

The active compounds may even be incorporated with excipients and used in the form of ingestible tablets, buccal tables, troches, capsules, elixirs, suspensions, syrups, wafers, and the like (see, for example, Mathiowitz *et al.*, Nature 1997 Mar 27;386(6623):410-4; Hwang *et al.*, Crit Rev Ther Drug Carrier Syst 1998;15(3):243-84; U. S. Patent 5,641,515; U. S. Patent 5,580,579 and U. S. Patent 5,792,451). Tablets, troches, pills, capsules and the like may also contain any of a variety of additional components, for example, a binder, such as gum tragacanth, acacia, cornstarch, or gelatin; excipients, such as dicalcium phosphate; a disintegrating agent, such as corn starch, potato starch, alginic acid and the like; a lubricant, such as magnesium stearate; and a sweetening agent, such as sucrose, lactose or saccharin may be added or a flavoring agent, such as peppermint, oil of wintergreen, or cherry flavoring. When the dosage unit form is a capsule, it may contain, in addition to materials of the above type, a liquid carrier. Various other materials may be present as coatings or to otherwise modify the physical form of the dosage unit. For instance, tablets, pills, or capsules may be coated with shellac, sugar, or both. Of course, any material used in preparing any dosage unit form should be pharmaceutically pure and substantially non-toxic in the amounts employed. In addition, the active compounds may be incorporated into sustained-release preparation and formulations.

Typically, these formulations will contain at least about 0.1% of the active compound or more, although the percentage of the active ingredient(s) may, of course, be

varied and may conveniently be between about 1 or 2% and about 60% or 70% or more of the weight or volume of the total formulation. Naturally, the amount of active compound(s) in each therapeutically useful composition may be prepared in such a way that a suitable dosage will be obtained in any given unit dose of the compound. Factors such as solubility, bioavailability, biological half-life, route of administration, product shelf life, as well as other pharmacological considerations will be contemplated by one skilled in the art of preparing such pharmaceutical formulations, and as such, a variety of dosages and treatment regimens may be desirable.

For oral administration the compositions of the present invention may alternatively be incorporated with one or more excipients in the form of a mouthwash, dentifrice, buccal tablet, oral spray, or sublingual orally-administered formulation. Alternatively, the active ingredient may be incorporated into an oral solution such as one containing sodium borate, glycerin and potassium bicarbonate, or dispersed in a dentifrice, or added in a therapeutically-effective amount to a composition that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants. Alternatively the compositions may be fashioned into a tablet or solution form that may be placed under the tongue or otherwise dissolved in the mouth.

In certain circumstances it will be desirable to deliver the pharmaceutical compositions disclosed herein parenterally, intravenously, intramuscularly, or even intraperitoneally. Such approaches are well known to the skilled artisan, some of which are further described, for example, in U. S. Patent 5,543,158; U. S. Patent 5,641,515 and U. S. Patent 5,399,363. In certain embodiments, solutions of the active compounds as free base or pharmacologically acceptable salts may be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions may also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations generally will contain a preservative to prevent the growth of microorganisms.

Illustrative pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of

sterile injectable solutions or dispersions (for example, see U. S. Patent 5,466,468). In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier  
5 can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and/or vegetable oils. Proper fluidity may be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and/or by the use of surfactants. The prevention of the action of microorganisms  
10 can be facilitated by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and  
15 gelatin.

In one embodiment, for parenteral administration in an aqueous solution, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In  
20 this connection, a sterile aqueous medium that can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage may be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will  
25 necessarily occur depending on the condition of the subject being treated. Moreover, for human administration, preparations will of course preferably meet sterility, pyrogenicity, and the general safety and purity standards as required by FDA Office of Biologics standards.



In another embodiment of the invention, the compositions disclosed herein may be formulated in a neutral or salt form. Illustrative pharmaceutically-acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like. Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective.

The carriers can further comprise any and all solvents, dispersion media, vehicles, coatings, diluents, antibacterial and antifungal agents, isotonic and absorption delaying agents, buffers, carrier solutions, suspensions, colloids, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions is contemplated. Supplementary active ingredients can also be incorporated into the compositions. The phrase "pharmaceutically-acceptable" refers to molecular entities and compositions that do not produce an allergic or similar untoward reaction when administered to a human.

In certain embodiments, the pharmaceutical compositions may be delivered by intranasal sprays, inhalation, and/or other aerosol delivery vehicles. Methods for delivering genes, nucleic acids, and peptide compositions directly to the lungs *via* nasal aerosol sprays has been described, *e.g.*, in U. S. Patent 5,756,353 and U. S. Patent 5,804,212. Likewise, the delivery of drugs using intranasal microparticle resins (Takenaga *et al.*, J Controlled Release 1998 Mar 2;52(1-2):81-7) and lysophosphatidyl-glycerol compounds (U. S. Patent 5,725,871) are also well-known in the pharmaceutical arts. Likewise, illustrative transmucosal drug delivery in the form of a polytetrafluoroethylene support matrix is described in U. S. Patent 5,780,045.

In certain embodiments, liposomes, nanocapsules, microparticles, lipid particles, vesicles, and the like, are used for the introduction of the compositions of the present invention into suitable host cells/organisms. In particular, the compositions of the present invention may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, or a nanoparticle or the like. Alternatively, compositions of the present invention can be bound, either covalently or non-covalently, to the surface of such carrier vehicles.

The formation and use of liposome and liposome-like preparations as potential drug carriers is generally known to those of skill in the art (see for example, Lasic, Trends Biotechnol 1998 Jul;16(7):307-21; Takakura, Nippon Rinsho 1998 Mar;56(3):691-5; Chandran *et al.*, Indian J Exp Biol. 1997 Aug;35(8):801-9; Margalit, Crit Rev Ther Drug Carrier Syst. 1995;12(2-3):233-61; U.S. Patent 5,567,434; U.S. Patent 5,552,157; U.S. Patent 5,565,213; U.S. Patent 5,738,868 and U.S. Patent 5,795,587, each specifically incorporated herein by reference in its entirety).

Liposomes have been used successfully with a number of cell types that are normally difficult to transfect by other procedures, including T cell suspensions, primary hepatocyte cultures and PC 12 cells (Renneisen *et al.*, J Biol Chem. 1990 Sep 25;265(27):16337-42; Muller *et al.*, DNA Cell Biol. 1990 Apr;9(3):221-9). In addition, liposomes are free of the DNA length constraints that are typical of viral-based delivery systems. Liposomes have been used effectively to introduce genes, various drugs, radiotherapeutic agents, enzymes, viruses, transcription factors, allosteric effectors and the like, into a variety of cultured cell lines and animals. Furthermore, the use of liposomes does not appear to be associated with autoimmune responses or unacceptable toxicity after systemic delivery.

In certain embodiments, liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles (also termed multilamellar vesicles (MLVs)).

Alternatively, in other embodiments, the invention provides for pharmaceutically-acceptable nanocapsule formulations of the compositions of the present

invention. Nanocapsules can generally entrap compounds in a stable and reproducible way (see, for example, Quintanar-Guerrero *et al.*, Drug Dev Ind Pharm. 1998 Dec;24(12):1113-28). To avoid side effects due to intracellular polymeric overloading, such ultrafine particles (sized around 0.1  $\mu\text{m}$ ) may be designed using polymers able to be degraded *in vivo*. Such particles can be made as described, for example, by Couvreur *et al.*, Crit Rev Ther Drug Carrier Syst. 1988;5(1):1-20; zur Muhlen *et al.*, Eur J Pharm Biopharm. 1998 Mar;45(2):149-55; Zambaux *et al.* J Controlled Release. 1998 Jan 2;50(1-3):31-40; and U.S. Patent 5,145,684.

### Cancer Therapeutic Methods

10 In further aspects of the present invention, the pharmaceutical compositions described herein may be used for the treatment of cancer, particularly for the immunotherapy of lung cancer. Within such methods, the pharmaceutical compositions described herein are administered to a patient, typically a warm-blooded animal, preferably a human. A patient may or may not be afflicted with cancer. Accordingly, the above  
15 pharmaceutical compositions may be used to prevent the development of a cancer or to treat a patient afflicted with a cancer. Pharmaceutical compositions and vaccines may be administered either prior to or following surgical removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs. As discussed above, administration of the pharmaceutical compositions may be by any  
20 suitable method, including administration by intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal, intradermal, anal, vaginal, topical and oral routes.

Within certain embodiments, immunotherapy may be active immunotherapy, in which treatment relies on the *in vivo* stimulation of the endogenous host immune system to react against tumors with the administration of immune response-modifying agents (such as polypeptides and polynucleotides as provided herein).  
25

Within other embodiments, immunotherapy may be passive immunotherapy, in which treatment involves the delivery of agents with established tumor-immune reactivity (such as effector cells or antibodies) that can directly or indirectly mediate

antitumor effects and does not necessarily depend on an intact host immune system. Examples of effector cells include T cells as discussed above, T lymphocytes (such as CD8<sup>+</sup> cytotoxic T lymphocytes and CD4<sup>+</sup> T-helper tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells and lymphokine-activated killer cells), B cells and

5 antigen-presenting cells (such as dendritic cells and macrophages) expressing a polypeptide provided herein. T cell receptors and antibody receptors specific for the polypeptides recited herein may be cloned, expressed and transferred into other vectors or effector cells for adoptive immunotherapy. The polypeptides provided herein may also be used to generate antibodies or anti-idiotypic antibodies (as described above and in U.S. Patent No.

10 4,918,164) for passive immunotherapy.

Effector cells may generally be obtained in sufficient quantities for adoptive immunotherapy by growth *in vitro*, as described herein. Culture conditions for expanding single antigen-specific effector cells to several billion in number with retention of antigen recognition *in vivo* are well known in the art. Such *in vitro* culture conditions typically use

15 intermittent stimulation with antigen, often in the presence of cytokines (such as IL-2) and non-dividing feeder cells. As noted above, immunoreactive polypeptides as provided herein may be used to rapidly expand antigen-specific T cell cultures in order to generate a sufficient number of cells for immunotherapy. In particular, antigen-presenting cells, such as dendritic, macrophage, monocyte, fibroblast and/or B cells, may be pulsed with

20 immunoreactive polypeptides or transfected with one or more polynucleotides using standard techniques well known in the art. For example, antigen-presenting cells can be transfected with a polynucleotide having a promoter appropriate for increasing expression in a recombinant virus or other expression system. Cultured effector cells for use in therapy must be able to grow and distribute widely, and to survive long term *in vivo*.

25 Studies have shown that cultured effector cells can be induced to grow *in vivo* and to survive long term in substantial numbers by repeated stimulation with antigen supplemented with IL-2 (*see*, for example, Cheever et al., *Immunological Reviews* 157:177, 1997).

Alternatively, a vector expressing a polypeptide recited herein may be introduced into antigen presenting cells taken from a patient and clonally propagated *ex vivo* for transplant back into the same patient. Transfected cells may be reintroduced into the patient using any means known in the art, preferably in sterile form by intravenous, intracavitary, intraperitoneal or intratumor administration.

Routes and frequency of administration of the therapeutic compositions described herein, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, the pharmaceutical compositions and vaccines may be administered by injection (*e.g.*, intracutaneous, intramuscular, intravenous or subcutaneous), intranasally (*e.g.*, by aspiration) or orally. Preferably, between 1 and 10 doses may be administered over a 52 week period. Preferably, 6 doses are administered, at intervals of 1 month, and booster vaccinations may be given periodically thereafter. Alternate protocols may be appropriate for individual patients. A suitable dose is an amount of a compound that, when administered as described above, is capable of promoting an anti-tumor immune response, and is at least 10-50% above the basal (*i.e.*, untreated) level. Such response can be monitored by measuring the anti-tumor antibodies in a patient or by vaccine-dependent generation of cytolytic effector cells capable of killing the patient's tumor cells *in vitro*. Such vaccines should also be capable of causing an immune response that leads to an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial or longer disease-free survival) in vaccinated patients as compared to non-vaccinated patients. In general, for pharmaceutical compositions and vaccines comprising one or more polypeptides, the amount of each polypeptide present in a dose ranges from about 25  $\mu$ g to 5 mg per kg of host. Suitable dose sizes will vary with the size of the patient, but will typically range from about 0.1 mL to about 5 mL.

In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome (*e.g.*, more frequent remissions, complete or partial, or longer disease-free survival) in treated patients

as compared to non-treated patients. Increases in preexisting immune responses to a tumor protein generally correlate with an improved clinical outcome. Such immune responses may generally be evaluated using standard proliferation, cytotoxicity or cytokine assays, which may be performed using samples obtained from a patient before and after treatment.

#### 5 Cancer Detection and Diagnostic Compositions, Methods and Kits

- In general, a cancer may be detected in a patient based on the presence of one or more lung tumor proteins and/or polynucleotides encoding such proteins in a biological sample (for example, blood, sera, sputum urine and/or tumor biopsies) obtained from the patient. In other words, such proteins may be used as markers to indicate the presence or absence of a cancer such as lung cancer. In addition, such proteins may be useful for the detection of other cancers. The binding agents provided herein generally permit detection of the level of antigen that binds to the agent in the biological sample. Polynucleotide primers and probes may be used to detect the level of mRNA encoding a tumor protein, which is also indicative of the presence or absence of a cancer. In general, a lung tumor sequence should be present at a level that is at least three fold higher in tumor tissue than in normal tissue

- There are a variety of assay formats known to those of ordinary skill in the art for using a binding agent to detect polypeptide markers in a sample. See, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1988. In general, the presence or absence of a cancer in a patient may be determined by (a) contacting a biological sample obtained from a patient with a binding agent; (b) detecting in the sample a level of polypeptide that binds to the binding agent; and (c) comparing the level of polypeptide with a predetermined cut-off value.

- In a preferred embodiment, the assay involves the use of binding agent immobilized on a solid support to bind to and remove the polypeptide from the remainder of the sample. The bound polypeptide may then be detected using a detection reagent that contains a reporter group and specifically binds to the binding agent/polypeptide complex. Such detection reagents may comprise, for example, a binding agent that specifically binds

to the polypeptide or an antibody or other agent that specifically binds to the binding agent, such as an anti-immunoglobulin, protein G, protein A or a lectin. Alternatively, a competitive assay may be utilized, in which a polypeptide is labeled with a reporter group and allowed to bind to the immobilized binding agent after incubation of the binding agent with the sample. The extent to which components of the sample inhibit the binding of the labeled polypeptide to the binding agent is indicative of the reactivity of the sample with the immobilized binding agent. Suitable polypeptides for use within such assays include full length lung tumor proteins and polypeptide portions thereof to which the binding agent binds, as described above.

10 The solid support may be any material known to those of ordinary skill in the art to which the tumor protein may be attached. For example, the solid support may be a test well in a microtiter plate or a nitrocellulose or other suitable membrane. Alternatively, the support may be a bead or disc, such as glass, fiberglass, latex or a plastic material such as polystyrene or polyvinylchloride. The support may also be a magnetic  
15 particle or a fiber optic sensor, such as those disclosed, for example, in U.S. Patent No. 5,359,681. The binding agent may be immobilized on the solid support using a variety of techniques known to those of skill in the art, which are amply described in the patent and scientific literature. In the context of the present invention, the term "immobilization" refers to both noncovalent association, such as adsorption, and covalent attachment (which  
20 may be a direct linkage between the agent and functional groups on the support or may be a linkage by way of a cross-linking agent). Immobilization by adsorption to a well in a microtiter plate or to a membrane is preferred. In such cases, adsorption may be achieved by contacting the binding agent, in a suitable buffer, with the solid support for a suitable amount of time. The contact time varies with temperature, but is typically between about 1  
25 hour and about 1 day. In general, contacting a well of a plastic microtiter plate (such as polystyrene or polyvinylchloride) with an amount of binding agent ranging from about 10 ng to about 10  $\mu$ g, and preferably about 100 ng to about 1  $\mu$ g, is sufficient to immobilize an adequate amount of binding agent.

Covalent attachment of binding agent to a solid support may generally be achieved by first reacting the support with a bifunctional reagent that will react with both the support and a functional group, such as a hydroxyl or amino group, on the binding agent. For example, the binding agent may be covalently attached to supports having an appropriate polymer coating using benzoquinone or by condensation of an aldehyde group on the support with an amine and an active hydrogen on the binding partner (*see, e.g.*, Pierce Immunotechnology Catalog and Handbook, 1991, at A12-A13).

In certain embodiments, the assay is a two-antibody sandwich assay. This assay may be performed by first contacting an antibody that has been immobilized on a solid support, commonly the well of a microtiter plate, with the sample, such that polypeptides within the sample are allowed to bind to the immobilized antibody. Unbound sample is then removed from the immobilized polypeptide-antibody complexes and a detection reagent (preferably a second antibody capable of binding to a different site on the polypeptide) containing a reporter group is added. The amount of detection reagent that remains bound to the solid support is then determined using a method appropriate for the specific reporter group.

More specifically, once the antibody is immobilized on the support as described above, the remaining protein binding sites on the support are typically blocked. Any suitable blocking agent known to those of ordinary skill in the art, such as bovine serum albumin or Tween 20™ (Sigma Chemical Co., St. Louis, MO). The immobilized antibody is then incubated with the sample, and polypeptide is allowed to bind to the antibody. The sample may be diluted with a suitable diluent, such as phosphate-buffered saline (PBS) prior to incubation. In general, an appropriate contact time (*i.e.*, incubation time) is a period of time that is sufficient to detect the presence of polypeptide within a sample obtained from an individual with lung cancer. Preferably, the contact time is sufficient to achieve a level of binding that is at least about 95% of that achieved at equilibrium between bound and unbound polypeptide. Those of ordinary skill in the art will recognize that the time necessary to achieve equilibrium may be readily determined by



assaying the level of binding that occurs over a period of time. At room temperature, an incubation time of about 30 minutes is generally sufficient.

Unbound sample may then be removed by washing the solid support with an appropriate buffer, such as PBS containing 0.1% Tween 20<sup>TM</sup>. The second antibody, which  
 5 contains a reporter group, may then be added to the solid support. Preferred reporter groups include those groups recited above.

The detection reagent is then incubated with the immobilized antibody-polypeptide complex for an amount of time sufficient to detect the bound polypeptide. An appropriate amount of time may generally be determined by assaying the level of binding  
 10 that occurs over a period of time. Unbound detection reagent is then removed and bound detection reagent is detected using the reporter group. The method employed for detecting the reporter group depends upon the nature of the reporter group. For radioactive groups, scintillation counting or autoradiographic methods are generally appropriate. Spectroscopic methods may be used to detect dyes, luminescent groups and fluorescent groups. Biotin  
 15 may be detected using avidin, coupled to a different reporter group (commonly a radioactive or fluorescent group or an enzyme). Enzyme reporter groups may generally be detected by the addition of substrate (generally for a specific period of time), followed by spectroscopic or other analysis of the reaction products.

To determine the presence or absence of a cancer, such as lung cancer, the  
 20 signal detected from the reporter group that remains bound to the solid support is generally compared to a signal that corresponds to a predetermined cut-off value. In one preferred embodiment, the cut-off value for the detection of a cancer is the average mean signal obtained when the immobilized antibody is incubated with samples from patients without the cancer. In general, a sample generating a signal that is three standard deviations above  
 25 the predetermined cut-off value is considered positive for the cancer. In an alternate preferred embodiment, the cut-off value is determined using a Receiver Operator Curve, according to the method of Sackett et al., *Clinical Epidemiology: A Basic Science for Clinical Medicine*, Little Brown and Co., 1985, p. 106-7. Briefly, in this embodiment, the cut-off value may be determined from a plot of pairs of true positive rates (*i.e.*, sensitivity)

and false positive rates (100%-specificity) that correspond to each possible cut-off value for the diagnostic test result. The cut-off value on the plot that is the closest to the upper left-hand corner (*i.e.*, the value that encloses the largest area) is the most accurate cut-off value, and a sample generating a signal that is higher than the cut-off value determined by this method may be considered positive. Alternatively, the cut-off value may be shifted to the left along the plot, to minimize the false positive rate, or to the right, to minimize the false negative rate. In general, a sample generating a signal that is higher than the cut-off value determined by this method is considered positive for a cancer.

In a related embodiment, the assay is performed in a flow-through or strip test format, wherein the binding agent is immobilized on a membrane, such as nitrocellulose. In the flow-through test, polypeptides within the sample bind to the immobilized binding agent as the sample passes through the membrane. A second, labeled binding agent then binds to the binding agent-polypeptide complex as a solution containing the second binding agent flows through the membrane. The detection of bound second binding agent may then be performed as described above. In the strip test format, one end of the membrane to which binding agent is bound is immersed in a solution containing the sample. The sample migrates along the membrane through a region containing second binding agent and to the area of immobilized binding agent. Concentration of second binding agent at the area of immobilized antibody indicates the presence of a cancer. Typically, the concentration of second binding agent at that site generates a pattern, such as a line, that can be read visually. The absence of such a pattern indicates a negative result. In general, the amount of binding agent immobilized on the membrane is selected to generate a visually discernible pattern when the biological sample contains a level of polypeptide that would be sufficient to generate a positive signal in the two-antibody sandwich assay, in the format discussed above. Preferred binding agents for use in such assays are antibodies and antigen-binding fragments thereof. Preferably, the amount of antibody immobilized on the membrane ranges from about 25 ng to about 1  $\mu$ g, and more preferably from about 50 ng to about 500 ng. Such tests can typically be performed with a very small amount of biological sample.

Of course, numerous other assay protocols exist that are suitable for use with the tumor proteins or binding agents of the present invention. The above descriptions are intended to be exemplary only. For example, it will be apparent to those of ordinary skill in the art that the above protocols may be readily modified to use tumor polypeptides to detect antibodies that bind to such polypeptides in a biological sample. The detection of such tumor protein specific antibodies may correlate with the presence of a cancer.

A cancer may also, or alternatively, be detected based on the presence of T cells that specifically react with a tumor protein in a biological sample. Within certain methods, a biological sample comprising CD4<sup>+</sup> and/or CD8<sup>+</sup> T cells isolated from a patient is incubated with a tumor polypeptide, a polynucleotide encoding such a polypeptide and/or an APC that expresses at least an immunogenic portion of such a polypeptide, and the presence or absence of specific activation of the T cells is detected. Suitable biological samples include, but are not limited to, isolated T cells. For example, T cells may be isolated from a patient by routine techniques (such as by Ficoll/Hypaque density gradient centrifugation of peripheral blood lymphocytes). T cells may be incubated *in vitro* for 2-9 days (typically 4 days) at 37°C with polypeptide (*e.g.*, 5 - 25 µg/ml). It may be desirable to incubate another aliquot of a T cell sample in the absence of tumor polypeptide to serve as a control. For CD4<sup>+</sup> T cells, activation is preferably detected by evaluating proliferation of the T cells. For CD8<sup>+</sup> T cells, activation is preferably detected by evaluating cytolytic activity. A level of proliferation that is at least two fold greater and/or a level of cytolytic activity that is at least 20% greater than in disease-free patients indicates the presence of a cancer in the patient.

As noted above, a cancer may also, or alternatively, be detected based on the level of mRNA encoding a tumor protein in a biological sample. For example, at least two oligonucleotide primers may be employed in a polymerase chain reaction (PCR) based assay to amplify a portion of a tumor cDNA derived from a biological sample, wherein at least one of the oligonucleotide primers is specific for (*i.e.*, hybridizes to) a polynucleotide encoding the tumor protein. The amplified cDNA is then separated and detected using techniques well known in the art, such as gel electrophoresis. Similarly, oligonucleotide

probes that specifically hybridize to a polynucleotide encoding a tumor protein may be used in a hybridization assay to detect the presence of polynucleotide encoding the tumor protein in a biological sample.

- To permit hybridization under assay conditions, oligonucleotide primers and
- 5 probes should comprise an oligonucleotide sequence that has at least about 60%, preferably at least about 75% and more preferably at least about 90%, identity to a portion of a polynucleotide encoding a tumor protein of the invention that is at least 10 nucleotides, and preferably at least 20 nucleotides, in length. Preferably, oligonucleotide primers and/or probes hybridize to a polynucleotide encoding a polypeptide described herein under
  - 10 moderately stringent conditions, as defined above. Oligonucleotide primers and/or probes which may be usefully employed in the diagnostic methods described herein preferably are at least 10-40 nucleotides in length. In a preferred embodiment, the oligonucleotide primers comprise at least 10 contiguous nucleotides, more preferably at least 15 contiguous nucleotides, of a DNA molecule having a sequence as disclosed herein. Techniques for
  - 15 both PCR based assays and hybridization assays are well known in the art (*see*, for example, Mullis et al., *Cold Spring Harbor Symp. Quant. Biol.*, 51:263, 1987; Erlich ed., *PCR Technology*, Stockton Press, NY, 1989).

- One preferred assay employs RT-PCR, in which PCR is applied in conjunction with reverse transcription. Typically, RNA is extracted from a biological
- 20 sample, such as biopsy tissue, and is reverse transcribed to produce cDNA molecules. PCR amplification using at least one specific primer generates a cDNA molecule, which may be separated and visualized using, for example, gel electrophoresis. Amplification may be performed on biological samples taken from a test patient and from an individual who is not afflicted with a cancer. The amplification reaction may be performed on several
  - 25 dilutions of cDNA spanning two orders of magnitude. A two-fold or greater increase in expression in several dilutions of the test patient sample as compared to the same dilutions of the non-cancerous sample is typically considered positive.

In another embodiment, the compositions described herein may be used as markers for the progression of cancer. In this embodiment, assays as described above for

the diagnosis of a cancer may be performed over time, and the change in the level of reactive polypeptide(s) or polynucleotide(s) evaluated. For example, the assays may be performed every 24-72 hours for a period of 6 months to 1 year, and thereafter performed as needed. In general, a cancer is progressing in those patients in whom the level of polypeptide or polynucleotide detected increases over time. In contrast, the cancer is not progressing when the level of reactive polypeptide or polynucleotide either remains constant or decreases with time.

Certain *in vivo* diagnostic assays may be performed directly on a tumor. One such assay involves contacting tumor cells with a binding agent. The bound binding agent may then be detected directly or indirectly via a reporter group. Such binding agents may also be used in histological applications. Alternatively, polynucleotide probes may be used within such applications.

As noted above, to improve sensitivity, multiple tumor protein markers may be assayed within a given sample. It will be apparent that binding agents specific for different proteins provided herein may be combined within a single assay. Further, multiple primers or probes may be used concurrently. The selection of tumor protein markers may be based on routine experiments to determine combinations that results in optimal sensitivity. In addition, or alternatively, assays for tumor proteins provided herein may be combined with assays for other known tumor antigens.

The present invention further provides kits for use within any of the above diagnostic methods. Such kits typically comprise two or more components necessary for performing a diagnostic assay. Components may be compounds, reagents, containers and/or equipment. For example, one container within a kit may contain a monoclonal antibody or fragment thereof that specifically binds to a tumor protein. Such antibodies or fragments may be provided attached to a support material, as described above. One or more additional containers may enclose elements, such as reagents or buffers, to be used in the assay. Such kits may also, or alternatively, contain a detection reagent as described above that contains a reporter group suitable for direct or indirect detection of antibody binding.

Alternatively, a kit may be designed to detect the level of mRNA encoding a tumor protein in a biological sample. Such kits generally comprise at least one oligonucleotide probe or primer, as described above, that hybridizes to a polynucleotide encoding a tumor protein. Such an oligonucleotide may be used, for example, within a PCR or hybridization assay. Additional components that may be present within such kits include a second oligonucleotide and/or a diagnostic reagent or container to facilitate the detection of a polynucleotide encoding a tumor protein.

The following Examples are offered by way of illustration and not by way of limitation.

10

### Example 1

#### PREPARATION OF LUNG TUMOR-SPECIFIC CDNA SEQUENCES USING DIFFERENTIAL DISPLAY RT-PCR

This example illustrates the preparation of cDNA molecules encoding lung tumor-specific polypeptides using a differential display screen.

Tissue samples were prepared from lung tumor and normal tissue of a patient with lung cancer that was confirmed by pathology after removal of samples from the patient. Normal RNA and tumor RNA was extracted from the samples and mRNA was isolated and converted into cDNA using a (dT)<sub>12</sub>AG (SEQ ID NO: 47) anchored 3' primer. Differential display PCR was then executed using a randomly chosen primer (SEQ ID NO: 48). Amplification conditions were standard buffer containing 1.5 mM MgCl<sub>2</sub>, 20 pmol of primer, 500 pmol dNTP and 1 unit of Taq DNA polymerase (Perkin-Elmer, Branchburg, NJ). Forty cycles of amplification were performed using 94 °C denaturation for 30 seconds, 42 °C annealing for 1 minute and 72 °C extension for 30 seconds. Bands that were repeatedly observed to be specific to the RNA fingerprint pattern of the tumor were cut out of a silver stained gel, subcloned into the pGEM-T vector (Promega, Madison, WI) and sequenced. The isolated 3' sequences are provided in SEQ ID NO: 1-16.

Comparison of these sequences to those in the public databases using the BLASTN program, revealed no significant homologies to the sequences provided in SEQ

ID NOs:1-11. To the best of the inventors' knowledge, none of the isolated DNA sequences have previously been shown to be expressed at a greater level in human lung tumor tissue than in normal lung tissue.

5

## Example 2

### USE OF PATIENT SERA TO IDENTIFY DNA SEQUENCES ENCODING

#### LUNG TUMOR ANTIGENS

This example illustrates the isolation of cDNA sequences encoding lung  
10 tumor antigens by expression screening of lung tumor samples with autologous patient sera.

A human lung tumor directional cDNA expression library was constructed  
employing the Lambda ZAP Express expression system (Stratagene, La Jolla, CA). Total  
RNA for the library was taken from a late SCID mouse passaged human squamous  
epithelial lung carcinoma and poly A+ RNA was isolated using the Message Maker kit  
15 (Gibco BRL, Gaithersburg, MD). The resulting library was screened using *E. coli*-  
absorbed autologous patient serum, as described in Sambrook et al., (*Molecular Cloning: A  
Laboratory Manual*, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY, 1989),  
with the secondary antibody being goat anti-human IgG-A-M (H + L) conjugated with  
alkaline phosphatase, developed with NBT/BCIP (Gibco BRL). Positive plaques  
20 expressing immunoreactive antigens were purified. Phagemid from the plaques was  
rescued and the nucleotide sequences of the clones was determined.

Fifteen clones were isolated, referred to hereinafter as LT86-1 - LT86-15.  
The isolated cDNA sequences for LT86-1 - LT86-8 and LT86-10 - LT86-15 are provided  
in SEQ ID NO:17-24 and 26-31, respectively, with the corresponding predicted amino acid  
25 sequences being provided in SEQ ID NOs:32-39 and 41-46, respectively. The determined  
cDNA sequence for LT86-9 is provided in SEQ ID NO:25, with the corresponding  
predicted amino acid sequences from the 3' and 5' ends being provided in SEQ ID NOs:40  
and 65, respectively. These sequences were compared to those in the gene bank as  
described above. Clones LT86-3, LT86-6 - LT86-9, LT86-11 - LT86-13 and LT86-15  
30 (SEQ ID NO: 19, 22-25, 27-29 and 31, respectively) were found to show some homology

to previously identified expressed sequence tags (ESTs), with clones LT86-6, LT86-8, LT86-11, LT86-12 and LT86-15 appearing to be similar or identical to each other. Clone LT86-3 was found to show some homology with a human transcription repressor. Clones LT86-6, 8, 9, 11, 12 and 15 were found to show some homology to a yeast RNA Pol II transcription regulation mediator. Clone LT86-13 was found to show some homology with a *C. elegans* leucine aminopeptidase. Clone LT86-9 appears to contain two inserts, with the 5' sequence showing homology to the previously identified antisense sequence of interferon alpha-induced P27, and the 3' sequence being similar to LT86-6. Clone LT86-14 (SEQ ID NO:30) was found to show some homology to the trithorax gene and has an "RGD" cell attachment sequence and a beta-Lactamase A site which functions in hydrolysis of penicillin. Clones LT86-1, LT86-2, LT86-4, LT86-5 and LT86-10 (SEQ ID NOs:17, 18, 20, 21 and 26, respectively) were found to show homology to previously identified genes. A subsequently determined extended cDNA sequence for LT86-4 is provided in SEQ ID NO:66, with the corresponding predicted amino acid sequence being provided in SEQ ID NO: 67.

Subsequent studies led to the isolation of five additional clones, referred to as LT86-20, LT86-21, LT86-22, LT86-26 and LT86-27. The determined 5' cDNA sequences for LT86-20, LT86-22, LT86-26 and LT86-27 are provided in SEQ ID NO: 68 and 70-72, respectively, with the determined 3' cDNA sequences for LT86-21 being provided in SEQ ID NO: 69. The corresponding predicted amino acid sequences for LT86-20, LT86-21, LT86-22, LT86-26 and LT86-27 are provided in SEQ ID NO: 73-77, respectively. LT86-22 and LT86-27 were found to be highly similar to each other. Comparison of these sequences to those in the gene bank as described above, revealed no significant homologies to LT86-22 and LT86-27. LT86-20, LT86-21 and LT86-26 were found to show homology to previously identified genes.

In further studies, a cDNA expression library was prepared using mRNA from a lung small cell carcinoma cell line in the lambda ZAP Express expression vector (Stratagene), and screened as described above, with a pool of two lung small cell carcinoma patient sera. The sera pool was adsorbed with *E. coli* lysate and human PBMC lysate was



added to the serum to block antibody to proteins found in normal tissue. Seventy-three clones were isolated. The determined cDNA sequences of these clones are provided in SEQ ID NO: 290-362. The sequences of SEQ ID NO: 289-292, 294, 296-297, 300, 302, 303, 305, 307-315, 317-320, 322-325, 327-332, 334, 335, 338-341, 343-352, 354-358, 360 and 362 were found to show some homology to previously isolated genes. The sequences of SEQ ID NO: 293, 295, 298, 299, 301, 304, 306, 316, 321, 326, 333, 336, 337, 342, 353, 359 and 361 were found to show some homology to previously identified ESTs.

### Example 3

#### 10 USE OF MOUSE ANTISERA TO IDENTIFY DNA SEQUENCES ENCODING LUNG TUMOR ANTIGENS

This example illustrates the isolation of cDNA sequences encoding lung tumor antigens by screening of lung tumor cDNA libraries with mouse anti-tumor sera.

A directional cDNA lung tumor expression library was prepared as described above in Example 2. Sera was obtained from SCID mice containing late  
15 passaged human squamous cell and adenocarcinoma tumors. These sera were pooled and injected into normal mice to produce anti-lung tumor serum. Approximately 200,000 PFUs were screened from the unamplified library using this antiserum. Using a goat anti-mouse IgG-A-M (H+L) alkaline phosphatase second antibody developed with NBT/BCIP (BRL  
20 Labs.), approximately 40 positive plaques were identified. Phage was purified and phagemid excised for 9 clones with inserts in a pBK-CMV vector for expression in prokaryotic or eukaryotic cells.

The determined cDNA sequences for 7 of the isolated clones (hereinafter referred to as L86S-3, L86S-12, L86S-16, L86S-25, L86S-36, L86S-40 and L86S-46) are  
25 provided in SEQ ID NO: 49-55, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 56-62, respectively. The 5' cDNA sequences for the remaining 2 clones (hereinafter referred to as L86S-30 and L86S-41) are provided in SEQ ID NO: 63 and 64. L86S-36 and L86S-46 were subsequently determined to represent the same gene. Comparison of these sequences with those in the public database as described

above, revealed no significant homologies to clones L86S-30, L86S-36 and L86S-46 (SEQ ID NO: 63, 53 and 55, respectively). L86S-16 (SEQ ID NO: 51) was found to show some homology to an EST previously identified in fetal lung and germ cell tumor. The remaining clones were found to show at least some degree of homology to previously identified human genes. Subsequently determined extended cDNA sequences for L86S-12, L86S-36 and L86S-46 are provided in SEQ ID NO: 78-80, respectively, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 81-83.

Subsequent studies led to the determination of 5' cDNA sequences for an additional nine clones, referred to as L86S-6, L86S-11, L86S-14, L86S-29, L86S-34, L86S-39, L86S-47, L86S-49 and L86S-51 (SEQ ID NO: 84-92, respectively). The corresponding predicted amino acid sequences are provided in SEQ ID NO: 93-101, respectively. L86S-30, L86S-39 and L86S-47 were found to be similar to each other. Comparison of these sequences with those in the gene bank as described above, revealed no significant homologies to L86S-14. L86S-29 was found to show some homology to a previously identified EST. L86S-6, L86S-11, L86S-34, L86S-39, L86S-47, L86S-49 and L86S-51 were found to show some homology to previously identified genes.

In further studies, a directional cDNA library was constructed using a Stratagene kit with a Lambda Zap Express vector. Total RNA for the library was isolated from two primary squamous lung tumors and poly A+ RNA was isolated using an oligo dT column. Antiserum was developed in normal mice using a pool of sera from three SCID mice implanted with human squamous lung carcinomas. Approximately 700,000 PFUs were screened from the unamplified library with *E. coli* absorbed mouse anti-SCID tumor serum. Positive plaques were identified as described above. Phage was purified and phagemid excised for 180 clones with inserts in a pBK-CMV vector for expression in prokaryotic or eukaryotic cells.

The determined cDNA sequences for 23 of the isolated clones are provided in SEQ ID NO: 126-148. Comparison of these sequences with those in the public database as described above revealed no significant homologies to the sequences of SEQ ID NO:

139 and 143-148. The sequences of SEQ ID NO: 126-138 and 140-142 were found to show homology to previously identified human polynucleotide sequences.

#### Example 4

##### USE OF MOUSE ANTISERA TO SCREEN LUNG TUMOR LIBRARIES

##### PREPARED FROM SCID MICE

This example illustrates the isolation of cDNA sequences encoding lung tumor antigens by screening of lung tumor cDNA libraries prepared from SCID mice with mouse anti-tumor sera.

A directional cDNA lung tumor expression library was prepared using a Stratagene kit with a Lambda Zap Express vector. Total RNA for the library was taken from a late passaged lung adenocarcinoma grown in SCID mice. Poly A<sup>+</sup> RNA was isolated using a Message Maker Kit (Gibco BRL). Sera was obtained from two SCID mice implanted with lung adenocarcinomas. These sera were pooled and injected into normal mice to produce anti-lung tumor serum. Approximately 700,000 PFUs were screened from the unamplified library with *E. coli*-absorbed mouse anti-SCID tumor serum. Positive plaques were identified with a goat anti-mouse IgG-A-M (H+L) alkaline phosphatase second antibody developed with NBT/BCIP (Gibco BRL). Phage was purified and phagemid excised for 100 clones with insert in a pBK-CMV vector for expression in prokaryotic or eukaryotic cells.

The determined 5' cDNA sequences for 33 of the isolated clones are provided in SEQ ID NO: 149-181. The corresponding predicted amino acid sequences for SEQ ID NO: 149, 150, 152-154, 156-158 and 160-181 are provided in SEQ ID NO: 182, 183, 186, 188-193 and 194-215, respectively. The clone of SEQ ID NO: 151 (referred to as SAL-25) was found to contain two open reading frames (ORFs). The predicted amino acid sequences encoded by these ORFs are provided in SEQ ID NO: 184 and 185. The clone of SEQ ID NO: 153 (referred to as SAL-50) was found to contain two open reading frames encoding the predicted amino acid sequences of SEQ ID NO: 187 and 216. Similarly, the clone of SEQ ID NO: 155 (referred to as SAL-66) was found to contain two open reading

frames encoding the predicted amino acid sequences of SEQ ID NO: 189 and 190. Comparison of the isolated sequences with those in the public database revealed no significant homologies to the sequences of SEQ ID NO: 151, 153 and 154. The sequences of SEQ ID NO: 149, 152, 156, 157 and 158 were found to show some homology to previously isolated expressed sequence tags (ESTs). The sequences of SEQ ID NO: 150, 155 and 159-181 were found to show homology to sequences previously identified in humans.

Using the procedures described above, two directional cDNA libraries (referred to as LT46-90 and LT86-21) were prepared from two late passaged lung squamous carcinomas grown in SCID mice and screened with sera obtained from SCID mice implanted with human squamous lung carcinomas. The determined cDNA sequences for the isolated clones are provided in SEQ ID NO: 217-237 and 286-289. SEQ ID NO: 286 was found to be a longer sequence of LT4690-71 (SEQ ID NO: 237). Comparison of these sequences with those in the public databases revealed no known homologies to the sequences of SEQ ID NO: 219, 220, 225, 226, 287 and 288. The sequences of SEQ ID NO: 218, 221, 222 and 224 were found to show some homology to previously identified sequences of unknown function. The sequence of SEQ ID NO: 236 was found to show homology to a known mouse mRNA sequence. The sequences of SEQ ID NO: 217, 223, 227-237, 286 and 289 showed some homology to known human DNA and/or RNA sequences.

In further studies using the techniques described above, one of the cDNA libraries described above (LT86-21) was screened with *E. coli*-absorbed mouse anti-SCID tumor serum. This serum was obtained from normal mice immunized with a pool of 3 sera taken from SCID mice implanted with human squamous lung carcinomas. The determined cDNA sequences for the isolated clones are provided in SEQ ID NO: 238-285. Comparison of these sequences with those in the public databases revealed no significant homologies to the sequences of SEQ ID NO: 253, 260, 277 and 285. The sequences of SEQ ID NO: 249, 250, 256, 266, 276 and 282 were found to show some homology to previously isolated expressed sequence tags (ESTs). The sequences of SEQ ID NO: 238-248, 251, 252, 254,

255, 257-259, 261-263, 265, 267-275, 278-281, 283 and 284 were found to show some homology to previously identified DNA or RNA sequences.

The expression levels of certain of the isolated antigens in lung tumor tissues compared to expression levels in normal tissues was determined by microarray technology. The results of these studies are shown below in Table 2, together with the databank analyses for these sequences.

TABLE 2

Clone	SEQ ID NO:	Description	LT+ F/N	SCC+M/N	Squa/ N	Adeno/ N
2LT-3	238	Unknown (KIAA0712)	2.2	3.8	3.3	-
2LT-6	239	Lactate DH B	2.3	3.8	4.1	-
2LT-22	240	Fumarate hydratase	-	3.0	-	-
2LT-26	242	CG1-39	-	-	12.8	-
2LT-31	243	ADH7	-	-	8.4	2.2
2LT-36	244	ADH7	-	2.4	2.0	-
2LT-42	245	HMG-CoA synthase	2.2	2.6	2.2	-
2LT-54	247	(Mus) ninein	-	2.1	-	-
2LT-55	248	Ubiquitin	2.2	-	2.5	2.0
2LT-57	249	Novel	2.1	2.9	2.4	-
2LT-58	250	Novel	2.3	4.0	2.9	-
2LT-59	251	Unknown KIAA0784	2.4	3.0	2.3	2.0
2LT_62	252	Nuc Pore Cmplx-ass pro TPR	-	-	-	2.1
2LT-70	256	Unknown KIAA0871	-	2.5	2.2	2.1
2LT-73	257	Mus polyadenylate-binding	-	2.0	-	-
2LT-76	259	Trans-Golgi p230	2.1	-	2.6	-
2LT-85	263	Ribosomal protein (LS29)	-	-	-	2.1
2LT-89	265	Unknown PAC212G6	-	2.0	-	-
2LT-98	268	Melanoma diff assoc pro 9	-	-	-	2.2
2LT-100	269	Mus Collagen alpha	-	-	-	2.1

Clone	SEQ ID NO:	Description	LT+ F/N	SCC+M/N	Squa/ N	Adeno/ N
		VI				
2LT-105	271	NY-CO-7 antigen	-	3.2	-	-
2LT-108	273	Unknown RG363M04	-	3.1	-	-
2LT-124	279	Galectin-9 (secreted)	2.3	2.7	2.0	-
2LT-126	280	L1 element L1.33 p40	2.5	-	3.1	-
2LT-128	282	Novel (kappa B-ras 2)	2.3+	-	20.4	2.5
2LT-133	284	Alpha II spectrin	-	2.3	-	-

LT+F/N = Lung Tumor plus Fetal tissue over Normal tissues

SC+M/N = Lung Small Cell carcinoma plus Metastatic over Normal tissues

Squa/N = Squamous lung tumor over Normal tissues

5 Aden/N = Adenocarcinoma over Normal tissues

Full-length sequencing studies on antigen 2LT-128 (SEQ ID NO: 282) resulted in the isolation of the full-length cDNA sequence provided in SEQ ID NO: 392. This amino acid sequence encoded by this full-length cDNA sequence is provided in SEQ ID NO: 393. This antigen shows 20-fold over-expression in squamous cell carcinoma and 10 2.5-fold over-expression in lung adenocarcinoma. This gene has been described as a potential ras oncogene (Fenwick et al. *Science*, 287:869-873, 2000).

Extended sequence information was obtained for clones 2LT-3 (SEQ ID NO:238), 2LT-26 (SEQ ID NO:242), 2LT-57 (SEQ ID NO: 249), 2LT-58 (SEQ ID NO:250), 2LT-98 (SEQ ID NO:268) and 2LT-124 (SEQ ID NO:279). The extended cDNA 15 sequences for these clones are set forth in SEQ ID NOs:428-433, respectively, encoding the polypeptide sequences set forth in SEQ ID NOs: 434-439, respectively.

## Example 5

## DETERMINATION OF TISSUE SPECIFICITY OF LUNG TUMOR POLYPEPTIDES

Using gene specific primers, mRNA expression levels for representative lung tumor polypeptides were examined in a variety of normal and tumor tissues using RT-PCR.

Briefly, total RNA was extracted from a variety of normal and tumor tissues using Trizol reagent. First strand synthesis was carried out using 2 µg of total RNA with SuperScript II reverse transcriptase (BRL Life Technologies) at 42 °C for one hour. The cDNA was then amplified by PCR with gene-specific primers. To ensure the quantitative nature of the RT-PCR, β-actin was used as an internal control for each of the tissues examined. 1 µl of 1:30 dilution of cDNA was employed to enable the linear range amplification of the β-actin template and was sensitive enough to reflect the differences in the initial copy numbers. Using these conditions, the β-actin levels were determined for each reverse transcription reaction from each tissue. DNA contamination was minimized by DNase treatment and by assuring a negative PCR result when using first strand cDNA that was prepared without adding reverse transcriptase.

mRNA Expression levels were examined in five different types of tumor tissue (lung squamous tumor from 3 patients, lung adenocarcinoma, prostate tumor, colon tumor and lung tumor), and different normal tissues, including lung from four patients, prostate, brain, kidney, liver, ovary, skeletal muscle, skin, small intestine, myocardium, retina and testes. L86S-46 was found to be expressed at high levels in lung squamous tumor, colon tumor and prostate tumor, and was undetectable in the other tissues examined. L86S-5 was found to be expressed in the lung tumor samples and in 2 out of 4 normal lung samples, but not in the other normal or tumor tissues tested. L86S-16 was found to be expressed in all tissues except normal liver and normal stomach. Using real-time PCR, L86S-46 was found to be over-expressed in lung squamous tissue and normal tonsil, with expression being low or undetectable in all other tissues examined.

## Example 6

## ISOLATION OF DNA SEQUENCES ENCODING LUNG TUMOR ANTIGENS

DNA sequences encoding antigens potentially involved in squamous cell lung tumor formation were isolated as follows.

5 A lung tumor directional cDNA expression library was constructed employing the Lambda ZAP Express expression system (Stratagene, La Jolla, CA). Total RNA for the library was taken from a pool of two human squamous epithelial lung carcinomas and poly A<sup>+</sup> RNA was isolated using oligo-dT cellulose (Gibco BRL, Gaithersburg, MD). Phagemid were rescued at random and the cDNA sequences of  
10 isolated clones were determined.

The determined cDNA sequence for the clone SLT-T1 is provided in SEQ ID NO: 102, with the determined 5' cDNA sequences for the clones SLT-T2, SLT-T3, SLT-T5, SLT-T7, SLT-T9, SLT-T10, SLT-T11 and SLT-T12 being provided in SEQ ID NO: 103-110, respectively. The corresponding predicted amino acid sequence for SLT-T1,  
15 SLT-T2, SLT-T3, SLT-T10 and SLT-T12 are provided in SEQ ID NO: 111-115, respectively. Comparison of the sequences for SLT-T2, SLT-T3, SLT-T5, SLT-T7, SLT-T9 and SLT-T11 with those in the public databases as described above, revealed no significant homologies. The sequences for SLT-T10 and SLT-T12 were found to show some homology to sequences previously identified in humans.

20 The sequence of SLT-T1 was determined to show some homology to a PAC clone of unknown protein function. The cDNA sequence of SLT-T1 (SEQ ID NO: 102) was found to contain a mutator (MUTT) domain. Such domains are known to function in removal of damaged guanine from DNA that can cause A to G transversions (see, for example, el-Deiry, W.S., 1997 *Curr. Opin. Oncol.* 9:79-87; Okamoto, K. et al. 1996 *Int. J. Cancer* 65:437-41; Wu, C. et al. 1995 *Biochem. Biophys. Res. Commun.* 214:1239-45;  
25 Porter, D.W. et al. 1996 *Chem. Res. Toxicol.* 9:1375-81). SLT-T1 may thus be of use in the treatment, by gene therapy, of lung cancers caused by, or associated with, a disruption in DNA repair.



In further studies, DNA sequences encoding antigens potentially involved in adenocarcinoma lung tumor formation were isolated as follows. A human lung tumor directional cDNA expression library was constructed employing the Lambda ZAP Express expression system (Stratagene, La Jolla, CA). Total RNA for the library was taken from a late SCID mouse passaged human adenocarcinoma and poly A+ RNA was isolated using the Message Maker kit (Gibco BRL, Gaithersburg, MD). Phagemid were rescued at random and the cDNA sequences of isolated clones were determined.

The determined 5' cDNA sequences for five isolated clones (referred to as SALT-T3, SALT-T4, SALT-T7, SALT-T8, and SALT-T9) are provided in SEQ ID NO: 116-120, with the corresponding predicted amino acid sequences being provided in SEQ ID NO: 121-125. SALT-T3 was found to show 98% identity to the previously identified human transducin-like enhancer protein TLE2. SALT-T4 appears to be the human homologue of the mouse H beta 58 gene. SALT-T7 was found to have 97% identity to human 3-mercaptopyruvate sulfurtransferase and SALT-T8 was found to show homology to human interferon-inducible protein 1-8U. SALT-T9 shows approximately 90% identity to human mucin MUC 5B.

cDNA sequences encoding antigens potentially involved in small cell lung carcinoma development were isolated as follows. cDNA expression libraries were constructed with mRNA from the small cell lung carcinoma cell lines NCIH69, NCIH128 and DMS79 (all available from the American Type Culture Collection, Manassas, VA) employing the Lambda ZAP Express expression system (Stratagene, La Jolla, CA). Phagemid were rescued at random and the cDNA sequences of 27 isolated clones were determined. Comparison of the determined cDNA sequences revealed no significant homologies to the sequences of SEQ ID NO: 372 and 373. The sequences of SEQ ID NO: 364, 369, 377, 379 and 386 showed some homology to previously isolated ESTs. The sequences of the remaining 20 clones showed some homology to previously identified genes. The cDNA sequences of these clones are provided in SEQ ID NO: 363, 365-368, 370, 371, 374-376, 378, 380-385 and 387-389, wherein SEQ ID NO: 363, 366-368, 370, 375, 376, 378, 380-382, 384 and 385 are full-length sequences.

Comparison of the cDNA sequence of SEQ ID NO: 372 indicated that this clone (referred to as 128T1) is a novel member of a family of putative seven pass transmembrane proteins. Specifically, using the computer algorithm PSORT, the protein was predicted to be a type IIIA plasma membrane seven pass transmembrane protein. A genomic clone was identified in the Genbank database which contained the predicted N-terminal 58 amino acids missing from the amino acid sequence encoded by SEQ ID NO: 372. The determined full-length cDNA sequence for the 128T1 clone is provided in SEQ ID NO: 390, with the corresponding amino acid sequence being provided in SEQ ID NO: 391.

The expression levels of certain of the isolated antigens in lung tumor tissues compared to expression levels in normal tissues was determined by microarray technology. The results of these studies are shown below in Table 3, together with the databank analyses for these sequences.

TABLE 3

Clone	SEQ ID NO:	Description	LT+ F/N	SCC+ M/N	Squa/ N	Adeno/N
DMS79-T1	363	STAT-ind inhib of cytokine	-	2.0	-	-
DMS79-T6	367	Neuronal cell death related	-	2.2	-	-
DMS79-T9	369	Novel	-	2.2	-	-
DMS79-T10	370	Ubiquitin carrier protein	-	3.9	2.2	-
DMS79-T11	371	HPV16E1 pro binding protein	-	2.1	-	-
128-T9	378	Elongation factor 1 alpha	-	2.7	-	-
128T11	380	Malate dehydrogenase	-	2.3	2.0	-
128-T12	381	Apurinic/apyrin endonuclease	-	5.4	-	-
NCIH69-T3	382	Sm-like protein CaSm	-	-	2.4	-
NCIH69-T6	384	Transcription factor BTF3a	-	2.5	-	-

- LT+F/N = Lung Tumor plus Fetal tissue over Normal tissues  
 SC+M/N = Lung Small Cell carcinoma plus Metastatic over Normal tissues  
 Squa/N = Squamous lung tumor over Normal tissues  
 5 Aden/N = Adenocarcinoma over Normal tissues

### Example 7

#### SYNTHESIS OF POLYPEPTIDES

- Polypeptides may be synthesized on a Perkin Elmer/Applied Biosystems  
 10 Division 430A peptide synthesizer using Fmoc chemistry with HPTU (O-Benzotriazole-  
 N,N,N',N'-tetramethyluronium hexafluorophosphate) activation. A Gly-Cys-Gly sequence  
 may be attached to the amino terminus of the peptide to provide a method of conjugation,  
 binding to an immobilized surface, or labeling of the peptide. Cleavage of the peptides  
 from the solid support may be carried out using the following cleavage mixture:  
 15 trifluoroacetic acid:ethanedithiol:thioanisole:water:phenol (40:1:2:2:3). After cleaving for  
 2 hours, the peptides may be precipitated in cold methyl-t-butyl-ether. The peptide pellets  
 may then be dissolved in water containing 0.1% trifluoroacetic acid (TFA) and lyophilized  
 prior to purification by C18 reverse phase HPLC. A gradient of 0%-60% acetonitrile  
 (containing 0.1% TFA) in water (containing 0.1% TFA) may be used to elute the peptides.  
 20 Following lyophilization of the pure fractions, the peptides may be characterized using  
 electrospray or other types of mass spectrometry and by amino acid analysis.

### Example 8

#### ISOLATION AND CHARACTERIZATION OF DNA SEQUENCES ENCODING LUNG

#### TUMOR ANTIGENS BY T-CELL EXPRESSION CLONING

25

Lung tumor antigens may also be identified by T cell expression cloning.  
 One source of tumor specific T cells is from surgically excised tumors from human  
 patients.

A non-small cell lung carcinoma was minced and enzymatically digested for several hours to release tumor cells and infiltrating lymphocytes (tumor infiltrating T cells, or TILs). The cells were washed in HBSS buffer and passed over a Ficoll (100%/75%/HBSS) discontinuous gradient to separate tumor cells and lymphocytes from non-viable cells. Two bands were harvested from the interfaces; the upper band at the 75%/HBSS interface contained predominantly tumor cells, while the lower band at the 100%/75%/HBSS interface contained a majority of lymphocytes. The TILs were expanded in culture, either in 24-well plates with culture media supplemented with 10 ng/ml IL-7 and 100 U/ml IL-2, or alternatively, 24-well plates that have been pre-coated with the anti-CD3 monoclonal antibody OKT3. The resulting TIL cultures were analyzed by FACS to confirm that a high percentage were CD8+ T cells (>90% of gated population) with only a small percentage of CD4+ cells.

In addition, non-small cell lung carcinoma cells were expanded in culture using standard techniques to establish a tumor cell line (referred to as LT391-06), which was later confirmed to be a lung carcinoma cell line by immunohistochemical analysis. This tumor cell line was transduced with a retroviral vector to express human CD80, and characterized by FACS analysis to confirm high expression levels of CD80, class I MHC and class II MHC molecules.

The ability of the TIL lines to specifically recognize autologous lung tumor was demonstrated by cytokine release assays (IFN- $\gamma$  and TNF- $\alpha$ ) as well as  $^{51}\text{Cr}$  release assays. Briefly, TIL cells from day 21 cultures were co-cultured with either autologous or allogeneic tumor cells, EBV-immortalized LCL, or control cell lines Daudi and K562, and the culture supernatant monitored by ELISA for the presence of cytokines. The TIL specifically recognized autologous tumor but not allogeneic tumor. In addition, there was no recognition of EBV-immortalized LCL or the control cell lines, indicating that the TIL lines are tumor specific and are potentially recognizing a tumor antigen presented by autologous MHC molecules.

The characterized tumor-specific TIL lines were expanded to suitable numbers for T cell expression cloning using soluble anti-CD3 antibody in culture with

irradiated EBV transformed LCLs and PBL feeder cells in the presence of 20 U/ml IL-2. Clones from the expanded TIL lines were generated by standard limiting dilution techniques. Specifically, TIL cells were seeded at 0.5 cells/well in a 96-well U bottom plate and stimulated with CD-80-transduced autologous tumor cells, EBV transformed LCL, and PBL feeder cells in the presence of 50 U/ml IL-2. The specificity of these clones for autologous tumor was confirmed by  $^{51}\text{Cr}$  microcytotoxicity and IFN- $\gamma$  bioassays.

These CTL clones were demonstrated to be HLA-B/C restricted by antibody blocking experiments. A representative CTL clone was tested on a panel of allogeneic lung carcinomas and it recognized both autologous tumor and a lung squamous cell carcinoma (936T). As the only class I MHC molecule shared among these tumors was HLA-Cw1203, this indicated that this was the restriction element used by the CTL. This finding was confirmed by the recognition of a number of allogeneic lung carcinomas transduced with a retroviral vector encoding HLA-Cw1203 by the CTL.

PolyA mRNA was prepared from a lung tumor cell line referred to as LT391-06 using Message Maker (Life Technologies; Rockville, MD). The subsequent steps involving cDNA synthesis were performed according to Life Technologies cloning manual (SuperScript Plasmid System for cDNA Synthesis and Plasmid Cloning). Modifications to the protocol were made as follows. At the adapter addition step, EcoRI-XmnI adapters (New England Biolabs; Beverly, MA) were substituted. Size fractionated cDNAs were ligated into the expression vector system HisMax A, B, C (Invitrogen; Carlsbad, CA) to optimize for protein expression in all three coding frames. Library plasmids were then aliquotted at approximately 100 CFU/well into a 96-well block for overnight liquid amplification. From these cultures, glycerol stocks were made and pooled plasmid was prepared by automated robot (Qiagen; Valencia, CA). The concentration of the plasmid DNA in each well of the library plates was determined to be approximately 150 ng/ul. Initial characterization of the cDNA expression library was performed by randomly sequencing 24 primary transformants and subjecting the resulting sequences to BLAST searches against available databases. The determined cDNA sequences are provided in SEQ ID NO: 443-480, with the results of the BLAST searches being provided in Table 4.

TABLE 4

Clone	SEQ ID NO:	GenBank Accession	Description
55163	458, 459		<i>Novel in Genbank</i>
55158	452		<i>Novel in Genbank</i>
<b>Homology to known sequences with unknown function</b>			
55153	443, 444	7018516	H. sapiens mRNA; cDNA DKFZp434M035
55154	445, 446	6437562	H. sapiens Chr 22q11 PAC Clone p393
55157	450, 451	2887408	H. sapiens KIAA0417 mRNA
55165	462, 463	3970871	H. sapiens HRIHFB2122 mRNA
<b>Homology to known sequences with known function</b>			
55155	447	7677405	H. sapiens F-box protein FBS (FBS)
55156	448, 449	3929584	H. sapiens EEN pseudogene
55161	454, 455	4503350	H. sapiens DNA (cytosine-5-)-methyltransferase 1 (DNMT1)
55162	456, 457	31220	ERK1 mRNA for protein serine/threonine kinase
55164	460, 461	6677666	H. sapiens RNA-binding protein (autoantigenic) (RALY)
55166	464, 465	3249540	H. sapiens ribonuclease P protein subunit p40 (RPP40)
55167	466, 467	7657497	H. sapiens renal tumor antigen (RAGE)
55168	468, 469	2873376	H. sapiens exportin t mRNA
55169	470, 471	3135472	H. sapiens Cre binding protein-like 2 mRNA
55171	474	4759151	H. sapiens spermine synthase (SMS)
55173	476	6688148	H. sapiens partial mRNA for NICE-3 protein
55174	477, 478	531394	Human transcriptional coactivator PC4
55175	479	6563201	H. sapiens translation initiation factor eIF-2b delta subunit
55176	480	29860	hCENP-Bgene, for centromere autoantigen B (CENP-B)
<b>Homology to Ribosomal Protein</b>			
55159	453	337494	Ribosomal protein L7a (surf 3) large subunit mRNA
55170	472, 473	4506648	H.sapiens mRNA for ribosomal protein L3
55172	475	388031	H. sapiens ribosomal protein L11

For T cell screening, approximately 80 ng of the library plasmid DNA and 80 ng of HLA-Cw1203 plasmid DNA was mixed with the lipid Fugene according to the manufacturers' instructions and transfected in duplicate into COS-7 cells. After incubation at 37 °C for 48 hours, the transfection mixture was removed and 10,000 LT391-06 CTL were added to each well in fresh media containing human serum.

The ability of T cells to recognize an antigen in the library was assessed by cytokine release after 6 hours (TNF-alpha, WEHI bio-assay) or after 24 hours (IFN-gamma, ELISA). Approximately  $2.0 \times 10^5$  clones (in plasmid pools of 100) were screened using this system in COS-7 cells. Three plasmid pools were identified (referred to as 14F10, 19A4, and 20E10) that were recognized by LT391-06 CTL. Transfection of these plasmid pools into COS-7 cells led to production of both IFN-gamma and TNF-alpha from the LT391-06 CTL at levels significantly above background. Pools 14F10, 19A4 and 20E10 were "broken down" into several hundred individual plasmid DNAs and retested. The sequences of 24 novel clones isolated from pool 14F10 are provided in SEQ ID NO: 481-511.

One plasmid (3D9) from pool 14F10, one plasmid from pool 20E10 and 5 plasmids (2A6, 2E11, 2F12, 3F4, 3H8) from pool 19A4 were capable of reconstituting T cell recognition. Sequencing of these plasmids led to the identification of a 7.8 kB cDNA insert (referred to as clone 14F10), a 2.2 kB cDNA insert (referred to as clone 19A4; SEQ ID NO:440), and a clone referred to as 20E10. The full-length cDNA sequence for 14F10 is provided in SEQ ID NO: 441. Clone 14F10 does not contain the first two "G" nucleotides found at the 5' end of 19A4, and the 3'-proximal 24 bp of 19A4 differ from the corresponding region of 14F10 (nucleotides 2145-2165). Furthermore, 3837 bp of 3' additional sequence was isolated for clone 14F10. The 5' terminal cDNA sequence (337 bp) of clone 20E10 is provided in SEQ ID NO: 442. 20E10 contains an additional 3 nucleotides (as compared to 19A4) at the 5'-most end. The additional sequence from the 5' end of clone 20E10 contains an "ATG" and therefore appears to contain the translational start site of a novel open reading frame. BLAST search analysis against the GenBank database identified these sequences as having significant homology with a truncated human cystine/glutamate transporter gene. Unlike the published sequence, however, clones 14F10

and 19A4 contain a unique 5' terminus consisting of 181 nucleotides. This novel sequence replaces the published 5' region and results in the removal of the reported initiating methionine (start codon) and an additional two amino acids of the reported transporter protein. Therefore, the translated product of clones 14F10 and 19A4 is different than the cystine/glutamate transporter protein. Furthermore, T cell recognition of other lung tumors demonstrates that this antigen is expressed by other tumors as well.

The epitope and amino acid sequence encoded within clones 19A4 and 14F10 which reconstitutes T cell recognition of anti-LT391-06 cells were mapped as follows. Cos-7 cells were transfected with 80 ng/well HLA-Cw1203 along with titrated amounts of cDNA encoding clone 19A4, a potential open reading frame located in the unique 5' terminus of 19A4, or the open reading frame from the cystine/glutamate (Cys-Glu) transporter gene, cloned into a eukaryotic expression vector and tested for stimulation of anti-LT391-06 T cells in a TNF assay. As a positive control Cos-7 cells were co-transfected with HLA-Cw1203 and the positive plasmid clone 19A4 described above. The Cys-Glu transporter expression construct was isolated by PCR using 5' and 3' primers specific for the known ORF of the transporter with 19A4 as template. In addition, each 5' primer contained a Kozak translation initiation site and starting methionine to drive translation of the polypeptide. CTL against LT391-06 did not recognize transfectants expressing the Cys-Glu transporter construct, but did recognize transfectants expressing 19A4 and the 5' ORF from 19A4.

In subsequent experiments, Cos-7 cells were co-transfected with 80 ng/well HLA-Cw1203 along with titrated amounts of DNA of transposition mutants F10 and C12, respectively, and tested for stimulation of anti-LT391-06 T cells in a TNF assay. As a positive control, Cos-7 cells were co-transfected with HLA-Cw1203 and clones of the 5' ORF of 19A4. Transposition mutants F10 and C12 were obtained by transposon-mediated mutation of the 14F10 clone and screening for insertion site by sequence analyses. The transposon of mutant F10 is inserted approximately 304 bp from the 5' EcoRI cloning site of the 14F10 cDNA. This mutation did not disrupt translation of the T cell epitope. By contrast, the transposon of mutant C12, which is inserted approximately 116 bp from the 5'



EcoRI cloning site of the 14F10 cDNA, was found to interrupt translation of the T cell epitope. Thus the epitope in 14F10 maps between these two transposon insertion sites. The amino acid sequence of the region between the C12 and F10 transposon insertion sites is provided in SEQ ID NO: 586.

- 5 A series of 11 overlapping 16-mer and 15-mer peptides for the region shown in SEQ ID NO: 586 were prepared and tested for stimulation of anti-LT391-06 cells, as determined by cytokine release in TNF and IFN- $\gamma$  assays. Only the peptide provided in SEQ ID NO: 587 (corresponding to residues 5-20 of SEQ ID NO: 586) stimulated cytokine release. These studies demonstrate that the HLA-Cw1203 restricted epitope of the LT391-  
10 06 antigen is contained within SEQ ID NO: 587.

#### Example 9

#### ISOLATION AND CHARACTERIZATION OF DNA SEQUENCES ENCODING LUNG TUMOR ANTIGENS BY PCR SUBTRACTION

- 15 This example describes the isolation and characterization of cDNA clones from a PCR subtracted expression library prepared from the human lung tumor cell line LT391-06 described above.

- Tester poly A mRNA was prepared from the cell line LT391-06 as described above. Driver poly A mRNA was isolated from a human acute T cell leukemia/T  
20 lymphocyte cell line (Jurkat) which is derived from non-lung cells and is not recognized by LT391-06 reactive T cells. The subtraction was performed according to the method of Clontech (Palo Alto, CA) with the following changes: 1) a second restriction digestion reaction of cDNA was completed using a pool of enzymes (MscI, PvuII, StuI and DraI). This was in addition to, and separate from, the Clontech recommended single restriction  
25 enzyme digestion with RsaI. Each restriction digest set was treated as a separate library to ensure that the final mixed library contained overlapping fragments. Thus, the epitope recognized by the T cells should be represented on a fragment within the library and not destroyed by the presence of a single restriction site within it. 2) The ratio of driver to tester cDNA was increased in the hybridization steps to increase subtraction stringency. To

analyze the efficiency of the subtraction, actin was PCR amplified from dilutions of subtracted, as well as unsubtracted, PCR samples. The second amplification step utilized primers that were modified from those normally used. Three nested PCR primers were engineered to contain a cleavable EcoRI site (not utilized during cloning) that was in one of three frames. Thus, secondary amplification with these primers resulted in products that could be ligated directly into the eukaryotic expression plasmid pcDNA4His/Max-Topo (Invitrogen). This resulted in the PCR subtracted and amplified fragments being represented in-frame somewhere within the library. Due to the mechanics of the subtraction only 50% of fragments will be in the correct orientation. The complexity and redundancy of the library was characterized by sequencing 96 randomly picked clones from the final pooled PCR subtraction expression library, referred to as LT391-06PCR. These sequences (SEQ ID NO: 512-581) were analyzed by comparison to sequences in publicly available databases (Table 5).

TABLE 5

Clone	SEQ ID NO:	GenBank Accession	Description
57235	532		<i>Novel in Genbank</i>
57255	547		<i>Novel in Genbank</i>
57264	554		<i>Novel in Genbank</i>
<b>Homology to known sequences with unknown function</b>			
57215	518	5689540	H. sapiens mRNA for KIAA1102 protein
57223	522	2341006	Human Xq13 3' end of PAC 92E23
57227	524	7022540	H. sapiens cDNA FLJ10480 fis, clone NT2RP2000126
57238	535	6807795	H. sapiens mRNA; cDNA DKFZp761G02121
57239	536	5757546	H. sapiens clone DJ0823F17
57243	539	7023805	H. sapiens cDNA FLJ11259 fis, clone PLACE1009045
57245	540	4884472	H. sapiens mRNA; cDNA DKFZp586O2223
57267	557	6808218	H. sapiens mRNA; cDNA DKFZp434O1519
57268	558	10040400	Sequence 12 from Patent WO9954460

Clone	SEQ ID NO:	GenBank Accession	Description
57270	560	7959775	H. sapiens PRO1489 mRNA
57271	561	4500158	H. sapiens mRNA; cDNA DKFZp586B0918
57281	567	6560920	H. sapiens clone RP11- 50107
57283	569	285962	Human mRNA for KIAA0108 gene
57285	570	7019813	H. sapiens cDNA FLJ20002 fis, clone ADKA01577
<b>Homology to known sequences with known function</b>			
57207	512	517176	H. sapiens YAP65 mRNA
57210	514	6841233	H. sapiens HSPC292 mRNA
57211	515	2606093	H. sapiens Cyr61 protein (CYR61) mRNA
57212	516	339648	Human thioredoxin (TXN) mRNA
57219	519	4504616	H. sapiens insulin- like growth factor binding protein 3 (IGFBP3)
57221	520	7274241	H. sapiens novel retinal pigment epithelial cell protein (NORPEG)
57222	521	189564	Human, plasminogen activator inhibitor- 1 gene
57228	525	4757755	H. sapiens annexin A2 (ANXA2)
57230	527	180800	Human alpha- 1 collagen type IV gene, exon 52
57232	529	6729061	H. sapiens clone RPC11- 98D12 from 7q31
57233	530	338391	Spermidine/ spermine N1- acetyltransferase
57234	531	7305302	H. sapiens NCK- associated protein 1 (NCKAP1)
57236	533	4929722	H. sapiens CGI- 127 protein
57242	538	4503558	H. sapiens epithelial membrane protein 1 (EMP1)
57248	541	183585	Human pregnancy- specific beta- glycoprotein c
57250	543	4759283	H. sapiens ubiquitin carboxyl- terminal esterase L1 (UCHL1)
57251	544	1236321	Human laminin gamma2 chain gene (LAMC2)
57253	545	213831	H. sapiens lysyl hydroxylase isoform 2 (PLOD2)
57254	546	536897	Human follistatin- related protein precursor mRNA
57257	548	339656	Human endothelial cell thrombomodulin
57258	549	190467	Human prion protein (PrP) mRNA

Clone	SEQ ID NO:	GenBank Accession	Description
57261	551	338031	Human serglycin gene
57262	552	178430	Human alphoid DNA (alphoid repetitive sequence)
57265	555	4502562	H. sapiens calpain, large polypeptide L2 (CAPN2)
57266	556	398163	H. sapiens mRNA for insulin- like growth factor binding protein- 3
57269	559	7262375	H. carboxylesterase 2 (intestine, liver) (CES2)
57272	562	467560	H. sapiens mRNA for cysteine dioxygenase type 1
57274	563	482664	H. sapiens annexin A3 (ANXA3)
57275	564	2281904	H. sapiens Bruton's tyr. kinase (BTK), alpha- D- galactosidase A (GLA)
57277	565	4557498	H. sapiens C- terminal binding protein 2 (CTBP2)
57282	568	189245	Human, NAD( P) H: menadione oxidoreductase mRNA
57287	571	28525	Human mRNA for amyloid A4 precursor of Alzheimer's disease
57288	572	4757755	H. sapiens annexin A2 (ANXA2)
57289	573	5729841	H. sapiens glyoxalase I (GLO1) mRNA
57290	574	6103642	H. sapiens F- box protein FBX3 mRNA
57295	576	182513	Human ferritin L chain mRNA
57299	579	37137	Human mRNA for thrombospondin
57301	580	179682	Human (clone A12) C4b- binding protein beta- chain
57302	581	6042205	H. sapiens membrane metallo- endopeptidase (neutral endopeptidase, enkephalinase, CALLA, CD10) (MME)
57213	517	2665791	H. sapiens caveolin- 2 mRNA
57259	550	2665791	H. sapiens caveolin- 2 mRNA
57225	523	179765	Human calcyclin gene
57229	526	179765	Human calcyclin gene
57237	534	186962	Human laminin B2 chain gene
57249	542	186962	Human laminin B2 chain gene
57231	528	4972626	H. sapiens caveolin 1 (CAV1) gene
57296	577	4972626	H. sapiens caveolin 1 (CAV1) gene
57297	578	4972626	H. sapiens caveolin 1 (CAV1) gene

Clone	SEQ ID NO:	GenBank Accession	Description
57240	537	266237	insulin- like growth factor binding protein 3
57292	575	184522	Human insulin- like growth factor- binding protein- 3 gene
57263	553	4504618	H. sapiens insulin- like growth factor binding protein 7 (IGFBP7)
57280	566	4504618	H. sapiens insulin- like growth factor binding protein 7 (IGFBP7)
<b>Homology to Ribosomal Protein</b>			
57209	513	337504	Human ribosomal protein S24 mRNA

## Example 10

## ISOLATION AND CHARACTERIZATION OF T CELL RECEPTORS FROM T CELL CLONES SPECIFIC FOR LUNG TUMOR ANTIGENS

5 This example describes the cloning and sequencing of T cell receptor (TCR) alpha and beta chains from a CD8 T cell clone specific for an antigen expressed by the lung tumor cell line LT391-06. T cells have a limited lifespan. Cloning of TCR chains and subsequent transfer would essentially enable infinite propagation of the T cell specificity. Cloning of tumor antigen TCR chains allows the transfer of the specificity into T cells  
 10 isolated from patients that share TCR MHC-restricting alleles. Such T cells can then be expanded and used in adoptive transfer techniques to introduce the tumor antigen specificity into patients carrying tumors that express the antigen (see, for example, Clay et al. *J. Immunol.* 163:507 (1999)).

Cytotoxic T lymphocyte (CTL) clones specific for the lung tumor cell line  
 15 LT391-06 were generated. Total mRNA from  $2 \times 10^6$  cells from 15 such clones was isolated using Trizol reagent and cDNA was synthesized using Ready-to-Go kits (Pharmacia). To determine Va and Vb sequences in these clones, a panel of Va and Vb subtype-specific primers was synthesized and used in RT-PCR reactions with cDNA  
 20 clones expressed a common Vb sequence that corresponded to the Vb13 subfamily. Using cDNA generated from one of the clones (referred to as 1105), the Va sequence expressed

was determined to be Va22. To clone the full TCR alpha and beta chains from clone 1105, primers were designed that spanned the initiator and terminator-coding TCR nucleotides. Standard 35-cycle RT-PCR reactions were established using cDNA synthesized from the CTL clone and the primers, with PWO (BMB) as the thermostable polymerase. The resultant specific bands (approximately 850 bp for the alpha chain and approximately 950 bp for the beta chain) were ligated into the PCR blunt vector (Invitrogen) and transformed into *E. coli*. *E. coli* transformed with plasmids containing the full-length alpha and beta chains were identified, and large scale preparations of the corresponding plasmids were generated. Plasmids containing full-length TCR alpha and beta chains were sequenced. The determined cDNA sequences for the alpha and beta chains are provided in SEQ ID NO: 583 and 582, respectively, with the corresponding amino acid sequences being provided in SEQ ID NO: 584 and 585, respectively.

#### Example 11

#### 15 CLONING OF cDNAs ENCODING LUNG SMALL CELL CARCINOMA ANTIGENS

Lung small cell carcinoma antigens were cloned by screening a small cell cDNA expression library with a mouse anti-SCID mouse serum. This antiserum was developed by growing lung small cell carcinoma cell lines NCIH69 and NCIH128 in SCID mice, removing SCID serum containing shed and secreted tumor antigens and immunizing normal mice with this serum. The library was constructed with mRNA from cell line NCIH128 in the lambda ZAP Express expression vector (Stratagene). The antiserum was adsorbed with *E. coli* lysate and human GAPDH protein and Ku autoantigens, and human PBMC lysate was added to the serum to block antibody to proteins found in normal tissue.

25 Sixty clones were isolated and the inserts of these clones were sequenced. The isolated clones and their respective sequence and clone identifiers are presented in Tables 6 and 7. The isolated clone sequences were compared to sequences in publically available databases. A summary of the Genbank homologies is found in Tables 6 and 7.

Those showing some degree of similarity with known sequences are described in Table 6, while those showing little or no similarity with known sequences are described in Table 7.

TABLE 6

SEQ ID NO.:	CLONE ID #	Genbank Homologies
589	54534	Homo sapiens mRNA for LAK-1
590	54536	Homo sapiens CGI-108 protein mRNA
591	54538	Human mRNA for HHR23A protein
592	54540	Homo sapiens chromosome 17, clone hRPC. 1030 0 14
593	55084	Homo sapiens homolog of rat elongation factor p18 (p18)
594	55086	Homo sapiens HSPC194 mRNA
595	54555	Homo sapiens accessory proteins BAP31/BAP29 (DXS1357E) mRNA
596	54557	Homo sapiens mesenchymal stem cell protein DSCD75 mRNA
597	54564	Homo sapiens prp28, U5 snRNP 100 kd protein (U5-100K) mRNA
599	55473	Homo sapiens uroporphyrinogen III synthase (congenital erythropoietic porphyria) (UROS)
600	55104	Homo sapiens carbonyl reductase (LOC51181)
601	55105	Homo sapiens membrane component, chromosome 11, surface marker 1 (M11S1)
602	55107	H.sapiens mRNA encoding GPI-anchored protein p137
604	55114	Homo sapiens mRNA; cDNA DKFZp56401716
605	55477	H.sapiens YB-1 gene promoter region
606	55482	Homo sapiens mRNA ; cDNA DKFZp434B0425
607	55483	Human Gu protein mRNA
608	55485	Homo sapiens 45kDa splicing factor mRNA
609	55487	Homo sapiens genomic DNA, chromosome 21q, section 72/105
610	55488	Homo sapiens chromosome 17, clone hCIT529110
612	55089	Homo sapiens scaffold attachment factor A (SAF-A) mRNA
613	55092	Homo sapiens density regulated protein drp1 mRNA
614	55093	H.sapiens mRNA encoding GPI-anchored protein p137
615	56926	Homo sapiens high-mobility group (nonhistone

SEQ ID NO.:	CLONE ID #	Genbank Homologies
		chromosomal) protein 17 (HMG17)
617	56944	Homo sapiens KBNA-2 co-activator (100kD) (p100), mRNA
619	55490	Homo sapiens death-associated protein 6 (DAXX) mRNA, and translated products.
620	55495	Homo sapiens mRNA for MEGF6
621	55504	Mus musculus hairy / enhancer of split 6 mRNA
624	56482	H.sapiens DNA from chromosome 19-cosmids R31158, R31874, & R28125, genomic seq.
626	56487	Human L23 mRNA for putative ribosomal protein
627	56488	Homo sapiens cDNA FLJ10526 fis, clone NT2RP2000931, highly similar to MATRIN 3
628	56490	Homo sapiens Sull1 isolog mRNA
630	56494	Homo sapiens mRNA; cDNA DKFZp564B167 (from clone DKFZp564B167)
631	56495	Homo sapiens 12p13.3 BAC RPC111-543P15 (Roswell Park Cancer Inst. Human BAC lib.)
632	56499	Human DNA-binding protein B (dbpB) gene, 3' end
633	56517	Homo sapiens esterase D mRNA
634	56952	Homo sapiens 14q32 Jagged2 gene, complete cds; and unknown gene
635	56953	Homo sapiens DNA polymerase zeta catalytic subunit (REV3L) mRNA
637	57139	Homo sapiens ribosomal protein, large, PO (RPLPO) mRNA
638	57078	Homo sapiens alpha-tubulin isoform 1 mRNA
640	57099	Homo sapiens uncharacterized hypothalamus protein HBEX2 mRNA
642	57105	Homo sapiens splicing factor, arginine/serine-rich 7 (35kD) (SFRS7)
643	57111	Human chromosome 14 DNA sequence
644	57117	Human DNA sequence from cosmid V857G56, between markers DXS366 and DXS87 on chromosome X contains ESTs
645	57121	Homo sapiens genomic DNA of 8p21.3-p22 anti-oncogene of hepatocellular colorectal and non-small cell lung cancer, segment 3/11
646	57124	H.sapiens MLN50 mRNA
647	57125	Homo sapiens calreticulin (CALR) , mRNA



Table 7

SEQ ID NO:.	CLONE ID #	Genbank Homologies
588	54533	Novel
598	55098	Novel
603	55108	Novel
611	55087	Novel (partial overlap of Unknown: Homo sapiens partial mRNA, clone c1-10e16)
616	56930	Novel
618	56945	Novel
622	55506	Novel / (136bp: Mus musculus mRNA for Rab24 protein)
623	56480	Novel
625	56484	Novel
629	56493	Novel
636	56959	Novel
639	57092	Novel
641	57100	Novel (last 120 bp: Unknown: Canine 21 kDa Signal peptase subunit mRNA)

In further studies, the expression levels of certain of these disclosed isolated antigens were compared to the expression levels in 36 normal tissues using microarray technology and computer analysis. These sequences were arrayed on Chip #7. The results of these studies are shown below in Table 8.

TABLE 8

Clone Name	Clone ID #	SEQ ID NO:	Squa/N	Aden/N	SC/N
LSCC2-1	54533	588	3	2	1
LSCC2-2	54534	589	5	3	5
LSCC2-4	54536	590	3	2	2
LSCC2-8	54540	592	0	3	2
LSCC2-18	55084	593	2	2	1
LSCC2-23	54555	595	2	3	3
LSCC2-25	54557	596	2	1	1
LSCC2-32	54564	597	2	3	2
LSCC2-48	55473	599	4	2	1
LSCC2-58	55104	600	3	5	2
LSCC2-61	55107	602	2	5	3

Clone Name	Clone ID #	SEQ ID NO:	Squa/N	Aden/N	SC/N
LSCC2-75	55483	607	2	4	2
LSCC2-79	55487	609	3	2	2
LSCC2-93	55089	612	5	4	4
LSCC2-121	55490	619	4	2	2
LSCC2-127	55495	620	2	4	1
LSCC2-137	55504	621	0	3	8
LSCC2-139	55506	622	3	4	1
LSCC2-161	56480	623	3	2	1
LSCC2-164	56482	624	2	4	2
LSCC2-171	56488	627	6	4	5
LSCC2-178	56494	670	3	5	3
LSCC2-191	56517	673	5	2	2

Squa/N = fold overexpression in squamous lung tumor versus normal tissues  
 Aden/N = fold overexpression in adenocarcinoma versus normal tissues  
 SC/N = fold overexpression in lung small cell carcinoma versus normal tissues

5

### Example 12

#### USE OF MOUSE ANTISERA TO IDENTIFY cDNA SEQUENCES ENCODING LUNG SMALL CELL CARCINOMA ANTIGENS

10

This example illustrates the isolation of cDNA sequences encoding lung small cell carcinoma antigens by screening a small cell carcinoma cell line cDNA library with mouse anti-SCID mouse sera.

A directional cDNA expression library was constructed with mRNA from small cell carcinoma cell line NCIH128 employing the Lambda ZAP Express expression system (Stratagene, La Jolla, CA). Sera was obtained from SCID mice containing human small cell carcinoma cell lines NCIH69 and NCIH128. The sera contains shed and secreted tumor antigens. These sera were pooled and injected into normal mice to produce anti-SCID mouse sera. The antiserum was absorbed with *E. coli* lysate, human GADPH protein and Ku autoantigens, and human PBMC lysate was added to the serum to block antibodies to proteins found in normal tissue.

20

Thirty-nine clones were isolated and the inserts of these clones were sequenced. The isolated clones and their respective sequence and clone identifier are presented in Table 9. The clone sequences were compared to sequences in publicly available databases (Geneseq, GenBank and huESTdb). A summary of these comparisons are found in Tables 10 and 11. Those showing some degree of homology with known sequences are described in Table 10, while those showing little or no similarity to known sequences are described in Table 11.

TABLE 9

CLONE NAME	SEQ. ID. NO:	CLONE ID #
LSCC-8	648	50664
LSCC-13	649	50669
LSCC-18	650	50673
LSCC-25	651	50680
LSCC-33	652	50685
LSCC-47	653	50699
LSCC-48	654	50700
LSCC-50	655	50702
LSCC-52	656	50704
LSCC-58	657	50710
LSCC-59	658	50711
LSCC-67	659	50719
LSCC-68	660	50720
LSCC-73	661	50725
LSCC-75	662	50727
LSCC-77	663	50729
LSCC-84	664	50736
LSCC-86	665	50738
LSCC-88	666	50740
LSCC-89	667	50741
LSCC-92	668	50744
LSCC-93	669	50745
LSCC-103	670	50754
LSCC-105	671	50756
LSCC-106	672	50757
LSCC-110	673	50761
LSCC-112	674	50763
LSCC-116	675	50767

CLONE NAME	SEQ. ID. NO:	CLONE ID #
LSCC-145	676	50775
LSCC-146	677	50776
LSCC-147	678	50777
LSCC-156	679	50786
LSCC-157	680	50787
LSCC-159	681	50789
LSCC-167	682	51003
LSCC-171	683	51007
LSCC-178	684	51014
LSCC-207	685	51304
LSCC-239	686	51568

TABLE 10

Seq. ID. No.	GenBank (ACCESS. #)	Description
648	D21094	Human mRNA for motor protein
652	NM_004487	Homo sapiens golgi autoantigen, golgin subfamily b, macrogolgin w/transmembrane signal
653	J04031	Human methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate synthetase mRNA
654	MN_007086	Homo sapiens AND-1 protein (AND-1), mRNA
657	J03483	Human chromogranin A mRNA
658	AF191340	Homo sapiens anaphase-promoting complex subunit 7 (APC7)
661	AC020663	Homo sapiens chromosome 16 clone RPC1-11_127120
662	D13388	Human mRNA for DnaJ protein homolog
663	AB014540	Homo sapiens mRNA for KIAA0640 protein, partial cds
666	NM_005898	Homo sapiens membrane component, chromosome 11, surface marker 1 (M11S1)
667	X75304	H.sapiens giantin mRNA
668	Z29067	H.sapiens AF-1p mRNA
669	AJ133129	H.sapiens mRNA for small glutamine-rich tetratricopeptide repeat containing protein
670	AF058918	Homo sapiens unknown mRNA
671	D89976	H.sapiens mRNA for 5-aminoimidazole-4-carboxamide ribonucleotide transformylase
672	NM_001539	Homo sapiens heat shock protein, DNAJ-like 2 (HSJ2) mRNA
673	AC020663	Homo sapiens chromosome 16 clone RPC1-11-127120
674	D21235	Human mRNA for HHR23A protein
676	MN_003804	Homo sapiens receptor (TNFRSF)-interacting serine-threonine

Seq. ID. No.	GenBank (ACCESS. #)	Description
		kinase 1 (RIPK1)
677	X76180	H.sapiens mRNA for lung amiloride sensitive Na <sup>+</sup> channel Protein
678	AB018330 U87803	Homo sapiens mRNA for KIAA0787 protein, partial cds Human putative ca <sup>2+</sup> /calmodulin-dependent protein kinase gene, 3' flanking region
679	L31610	Homo sapiens (clone cori-1c15) S29 ribosomal protein mRNA
680	Z83840	Human DNA sequence from clone CTA-216E10 on chromosome 22 contains the NHP2L1 gene for non-histone chromosome protein 2
682	D14696	Human mRNA for KIAA0108 gene
683	Z47087	H.sapiens mRNA for RNA polymerase II elongation factor-like protein
684	Z83840	Human DNA sequence from clone CTA-216E10 on chromosome 22 contains the NHP2L1 gene
685	U01923	Human BTK region clone ftp-3 mRNA

TABLE 11

Seq. ID. No.	GenBank (ACCESS. #)	Description
649		Novel
650	AC005023	Unknown: Homo sapiens BAC clone GS1-421I3 from Xq25-q26
651		Novel
655	AC007199	Unknown: Homo sapiens chromosome 5 BAC clone 111n13
656	AC005988	Unknown: Homo sapiens chromosome 17, clone hRPK.299 G 24
659	AK001695	Unknown: Homo sapiens cDNA FLJ10833 fis, clone NT2RP4001206, moderately similar to Drosophila melanogaster strawberry notch mRNA
660	AK001722	Unknown: Homo sapiens cDNA FLJ10860 fis, clone NT2RP4001568, weakly similar to ZINC FINGER PROTEIN GCS1
664	AK001925	Unknown: Homo sapiens cDNA FLJ11063 fis, clone PLACE1004814, weakly similar to SPLICING FACTOR, ARGININE/SERINE-RICH 4
665		Novel
675	(AJ131096)	Novel (1 to 103 bp is Picea abies microsatellite RNA), clone PAAG2
681	AP001065	Unknown: Homo sapiens genomic DNA, chromosome 21, clone:KB68A7, MX-D21S171 region
686		Novel

In further studies, the expression levels of certain of these disclosed isolated antigens were compared to the expression levels in 36 normal tissues using microarray technology and computer analysis. These sequences were arrayed on Chip #7. The results of these studies are shown below in Table 12.

5

TABLE 12

Clone Name	Clone ID #	SEQ ID NO:	Squa/N	Aden/N	SC/N
LSCC-8	50664	648	4	3	2
LSCC-13	50669	649	2	4	0
LSCC-59	50711	658	4	2	3
LSCC-84	50736	664	6	3	4
LSCC-86	50738	665	1	4	0
LSCC-88	50740	666	2	3	4
LSCC-92	50744	668	3	1	1
LSCC-105	50756	671	4	3	2
LSCC-106	50757	672	4	3	1
LSCC-110	50761	673	8	3	4
LSCC-146	50776	677	3	1	1
LSCC-147	50777	678	5	2	3
LSCC-156	50786	679	4	2	2
LSCC-159	50789	681	2	2	1
LSCC-171	51007	683	2	1	1
LSCC-207	51304	685	3	4	3
LSCC-239	51568	686	4	3	2

Squa/N = Squamous lung tumor versus Normal tissues

Aden/N = Adenocarcinoma over versus tissues

10 SC/N = Lung Small Cell carcinoma versus Normal tissues

### Example 13

#### USE OF MOUSE ANTISERA TO IDENTIFY cDNA SEQUENCES ENCODING LUNG SMALL CELL CARCINOMA ANTIGENS

15

This example illustrates the isolation of cDNA sequences encoding lung small cell carcinoma antigens by screening a small cell carcinoma cell line cDNA library with mouse anti-SCID mouse sera.

A directional cDNA expression library was constructed with mRNA from a SCID-passaged human lung cancer tumor DMS79 employing the Lambda ZAP Express expression system (Stratagene, La Jolla, CA). Sera was obtained from SCID mice containing the human lung cancer tumors DMS79 and NCIH688. The sera contains shed and secreted tumor antigens. These sera were pooled and injected into normal mice to produce anti-SCID mouse sera. The antiserum was absorbed with *E. coli* lysate, human GADPH protein and Ku autoantigens, and human PBMC lysate was added to the serum to block antibodies to proteins found in normal tissue.

Forty-one clones were isolated and the inserts of these clones were sequenced. The isolated clones and their respective sequence identifiers are presented in Table 13. The clone sequences were compared to sequences in publicly available databases. A summary of these comparisons are found in Tables 14 and 15. Those showing some degree of similarity with known sequences are described in Table 14, while those showing little or no similarity to known sequences are found in Table 15.

15

TABLE 13

CLONE NAME	SEQ. ID. NO.:	CLONE ID #
DMS-3	687	48564
DMS-8	688	48567
DMS-9	689	48568
DMS-12	690	48571
DMS-14	691	45572
DMS-25	692	48578
DMS-35	693	48583
DMS-38	694	48584
DMS-39	695	48585
DMS-47	696	49059
DMS-50	697	49061
DMS-53	698	49065
DMS-61	699	49070
DMS-63	700	49072
DMS-64	701	49073
DMS-67	702	49076
DMS-75	703	50793
DMS-76	704	50794

CLONE NAME	SEQ. ID. NO.:	CLONE ID #
DMS-79	705	50797
DMS-84	706	50800
DMS-93	707	50805
DMS-126	708	50984
DMS-129	709	50986
DMS-139	710	51065
DMS-151	711	51070
DMS-164	712	51078
DMS-168	713	51080
DMS-175	714	51084
DMS-193	715	51095
DMS-199	716	51099
DMS-200	717	51100
DMS-204	718	51103
DMS-214	719	51112
DMS-218	720	51113
DMS-221	721	51116
DMS-232	722	51123
DMS-253	723	51212
DMS-270	724	51220
DMS-275	725	51224
DMS-289	726	51234
DMS-296	727	51239

TABLE 14

SEQ ID NO:	GenBank
687	KIAA0013:cDNA from Hu. BM myeloblast line
688	Hu. Homolog Mu. LLRep3, sim. To ribosomal S2
689	KIAA0769, Hu. brain protein
690	Hu. Thymidylate kinase (CDC9), regul'n
691	Hu. Ku automimmune Ag; Nuc. Fctr. IV
692	Hu. Polyubiquitin UbC
693	Hu. FLJ20423 fis (signet-ring cell carc. Celline)
694	KIAA0640, SWAP-70 (Hu, brain protein)
695	Human radixin (cytoskeletal)
696	Hu. Ribosomal protein L13a
697	Hu. trk oncogene, cytoskltl. Tropomyosin
698	DKFZp586K2120 (uterus) KIAA0784 (brain)
699	Hu. Chromogranin A (parathyr. Secrtry. Pro. 1)
700	Hu. Glutathione-S-transferase homolog
701	Hu. lactate dehydrogenase-A



SEQ ID NO:	GenBank
702	Hu. GPI-anchored membr. Pro. p137
704	Hu. HMG-17
705	Hu. Ubiquitin C-terminal hydrolase (UHX1)
706	Hu. Cosmid 25, PAC clone RP5-901A4
707	Hu. lactate dehydrogenase B
708	Hu. NuMA gene
709	Hu. KIAA0008 gene
710	Hu. BCL2/adenovirus E1B pro.2 (BNIP2)
711	Hu. Unactive progesterone receptor P23
712	Hu. alpha II spectrin
713	Hu. Transcriptional coactivator ALY
714	Hu. DnaJ Heat Shock homolog
715	Hu. mitoch. Or Replication
716	Hu. Ornithine decarboxylase antizyme (brain)
717	Hu. Deoxycytidine kinase
718	Hu. Fumarase
719	Hu. 80K-H protein (kinase C substrate)
721	Hu. Neuro-d4 (rat) homolog
722	Hu. Sodium/glucose cotransporter, repeat
724	Hu. Zinc finger protein ZNF226
725	Hu. Jumping transloc'n brkpt. Gene
726	Hu. M-phase phosphoprotein
727	Hu. Peroxisomal signal receptor 1

TABLE 15

SEQ ID NO:	GenBank
703	Novel
720	Novel (ALU?)
723	Novel

## Example 14

5

## ANALYSIS OF cDNA EXPRESSION USING MICROARRAY TECHNOLOGY

In additional studies, four clones obtained in Example 13 were found to be overexpressed in specific tumor tissues as determined by microarray analysis. Using this approach, cDNA sequences are PCR amplified and their mRNA expression profiles in

tumor and normal tissues were examined using cDNA microarray technology essentially as described (Shena *et al.*, 1995). In brief, the clones are arrayed onto glass slides as multiple replicas, with each location corresponding to a unique cDNA clone (as many as 5500 clones can be arrayed on a single slide, or chip). Each chip is hybridized with a pair of cDNA probes that are fluorescence-labeled with Cy3 and Cy5, respectively. Typically, 1 µg of polyA<sup>+</sup> RNA is used to generate each cDNA probe. After hybridization, the chips are scanned and the fluorescence intensity recorded for both Cy3 and Cy5 channels. There are multiple built-in quality control steps. First, the probe quality is generally monitored using a panel of ubiquitously expressed genes. Secondly, the control plate also can include yeast DNA fragments of which complementary RNA may be spiked into the probe synthesis for measuring the quality of the probe and the sensitivity of the analysis. Currently, the technology offers a sensitivity of about 1 in 100,000 copies of mRNA. Finally, the reproducibility of this technology can be ensured by including duplicated control cDNA elements at different locations.

The extended predicted full length sequences for partial sequences of clones, DMS39, DMS126, DMS218 and DMS253 (previously isolated in Example 13) were obtained from the GenBank databases after database searches using the original partial cDNA sequences as the query. The predicted full length sequences for the cloned cDNA sequence for clones DMS39, DMS126, DMS218 and DMS253 are provided in SEQ ID NO:728-731, respectively. SEQ ID NO:728-731 were analyzed by comparison to sequences in the publicly available databases. A summary of these comparisons is presented in Table 16.

TABLE 16

SEQ ID NO:	Clone Name	Blastn
728	DMS-39	Human radixin
729	DMS-126	Human nuclear mitotic apparatus protein
730	DMS-218	Hu. cDNA: FLJ21840 fis; XPMC2
731	DMS-253	Hu. mRNA for KIAA1582 protein

## Example 15

## ANALYSIS OF CDNA EXPRESSION USING MICROARRAY TECHNOLOGY

5 In an additional study, a clone obtained in Example 12 was found to be overexpressed in specific tumor tissues as determined by microarray analysis. Using this approach, the cDNA sequence is PCR amplified and its mRNA expression profiles in tumor and normal tissues was examined using cDNA microarray technology as described in Example 13. Microarray analysis showed the cDNA for LSCC-86 is strongly overexpressed in small cell carcinoma cell line HTB 173; moderately overexpressed in atypical carcinoid METs, adenocarcinoma lung tumors and squamous lung tumors; and 10 slightly overexpressed in primary small cell. This cDNA is also strongly overexpressed in pituitary gland; moderately overexpressed in brain and adrenal gland; and slightly overexpressed in skeletal muscle.

15 Clone LSCC-86 was originally isolated in Example 12 and a partial sequence of this insert is provided in SEQ ID NO:665. An extended sequence was obtained by PCR sequencing using internal primer sequences designed from the partial cDNA sequence of clone LSCC-86. This extended sequence represents the full-length sequence for the cloned cDNA sequence of clone LSCC-86. The determined full length sequence for LSCC-86 is provided in SEQ ID NO:732. SEQ ID NO:732 was analyzed by comparison to sequences in the publicly available databases. Database searches showed no 20 homology in GenBank, seven ESTs (3 lung tumor and 4 uncatagorized hits) in the human EST database, and no homology in Blastx. Three open reading frames (ORFs) were identified. A first that encodes a protein with a sequence of 50 amino acid residues (SEQ ID NO:733) which is fused to LacZ. A second that encodes a protein with a sequence of 76 amino acids residues (SEQ ID NO:734) which shows no homology in the databases. A 25 third that encodes a protein with a sequence of 74 amino acid residues (SEQ ID NO:735) which also shows no homology in the databases. However, a motif search of SEQ ID NO:735 shows a possible small cytokine signature.

From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

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